THE CLINICAL SIGNIFICANCE OF SERUM AMYLOID A AND THE EFFECTS OF CORTICOSTEROIDS ON ITS PRODUCTION BY HEPG2 CELLS

By

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THE CLINICAL SIGNIFICANCE OF SERUM AMYLOID A AND THE EFFECTS OF CORTICOSTEROIDS ON ITS PRODUCTION BY HEPG2 CELLS

By

James W. Smith

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Under the Supervision of Dr. Thomas L. McDonald

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THE CLINICAL SIGNIFICANCE OF SERUM AMYLOID A AND THE EFFECTS OF CORTICOSTEROID ON ITS PRODUCTION BY HEPG2 CELLS

James W. Smith, Ph.D.

University of Nebraska, 1992

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Serum amyloid A (SAA) and C-reactive protein (CRP) are produced by the liver in response to inflammation. Both are indicators of inflammation, and CRP is much used in that regard. These studies' purpose was to develop and characterize standards and an assay for measuring SAA, determine the clinical utility of measuring SAA levels in cystic fibrosis patients, and translate the findings from patient studies into an in vitro system. A two-step protocol for purifying SAA from serum was developed, employing hydrophobic interaction chromatography eluted with ethanol followed by gel filtration under dissociating conditions. This protocol yielded a 56% recovery of SAA, >90% pure, suitable for used as a standard and for further biological studies. An ELISA for determining SAA concentrations in serum or tissue culture fluid, utilizing to monoclonal antibodies, was developed and characterized. Intra- and interassay coefficients of variation averaged 4.9% and 7.8% respectively. The assay was useful for measuring SAA concentrations in serum from five to 500 μg/ml; assay sensitivity was 2.5 ng/ml. SAA and CRP concentrations correlated strongly (r=0.80) in non-corticosteroid treated CF patients, but poorly (r=0.35) in corticosteroid treated (CFS) patients. SAA was a more sensitive measure of inflammation in CF patients, particularly ir CFS patients, and correlated with pulmonary function tests. A disparate production of SAA compare to CRP observed in CFS patients was explored

using HepG2 cells stimulated to produce CRP and SAA. HepG2 cells were stimulated with conditioned media from monocytes activated with killed P. aeruginosa or E. coli lipopolysaccharide, or with "cytokine soups" containing various combinations and concentrations of recombinant human (rh) cytokines. The corticosteroid prednisolone inhibited production of IL-1α and IL-1β by monocytes, but caused a three- to five-fold increase in production of SAA from HepG2 cells stimulated with monocyte conditioned media or with "cytokine soups". Prednisolone did not affect production of CRP by HepG2 cells. These observations offer a tenable explanation for the disparate production of SAA compared to CRP observed in CFS patients.

PREFACE

The results of my research efforts are described within a series of four manuscripts which have been published or are in press. Accordingly, this dissertation is organized around these manuscripts in the following manner: First, a general introduction describing the acute phase response and the production of the acute phase proteins, especially serum amyloid A (SAA) and C-reactive protein (CRP). Some immunomodulatory activities associated with these acute phase proteins are also described. Following this general introduction are three introductory sections which briefly discuss the rationale and pertinent background information for each manuscript. The Specific Aims and Results and Discussion are followed by a brief Summary, the Conclusions and a short section, Speculation on the Role of SAA, which ends this first section of the dissertation. The four manuscripts are then appended. The first section is not intended to be inclusive, and should be read in context with the four appended manuscripts.

The credit for the achievements noted within this dissertation belong largely to my family - my wife, Carie, and three sons, Stephen, Matthew, and Christopher. Their love, support, understanding, and humor have made it possible to pursue these endeavors. I love all of you.

Drs. Tom McDonald, Dave Crouse, Sam Pirruccello, Janos Luka and John Colombo - my graduate committee - you have been wonderful. Helpful, meaningful discussions and advice have given me immeasurable help in these studies. You were always available when needed, and always willing to help when asked. Thank you. Tom - your guidance has been especially helpful. The many meaningful discussions concerning the clinical implications of this research have made me aware of the link between the

research and the clinical lab.

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This dissertation is dedicated to my father-in-law, Richard Horsens, who has shown me what inner strength and commitment are.

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INTRODUCTION

Acute phase response

Inflammation induces both local and systemic reactions. Among the locally occurring responses are primary events such as vasodilation and the release of a number of cellular constituents, including lysosomal enzymes and prostaglandins. Local secondary reactions are quite diverse and include such events as the influx of white blood cells, the respiratory burst of neutrophils, chemotaxis, the production of leukotrienes and arachidonic acid metabolites, the activation of macrophages and monocytes, and the secretion of peptide hormones and cytokines. Systemic responses include fever, pain, negative nitrogen balance, and the acute phase response of the liver (Schultz and Arnold, 1990; Kushner and Mackiewicz, 1987; Kushner et al., 1989).

The acute phase response occurs without regard to the source or cause of the inflammation, whether that be physical or chemical tissue damage, viral, bacterial or parasitic infection, or neoplastic growth (Kushner and Mackiewicz, 1987; Kushner et al., 1989). Changes occur in the plasma concentrations of a group of proteins known as the acute phase proteins. These proteins are divided into two groups, dependent upon whether their plasma concentrations increase or decrease during an acute phase process. Those which decrease in concentration during the course of the inflammatory process, i.e., the negative acute phase proteins, are

characterized by serum albumin, α_2-microglobulin, and vitamin D-binding protein (Fey and Gauldie, 1990). Those which increase in concentration during the course of the inflammatory process, i.e., the positive acute phase proteins, can be divided into three groups based on the order of magnitude of increase seen: (a) those which increase in concentration approximately 50%, such as ceruloplasmin and the complement components C3 and C4, (b) those which increase in concentration two- to four-fold, such as α_1-acid glycoprotein (AAG), α_1-anti-trypsin, haptoglobin, and fibrinogen, and (c) those which increase in concentration by several hundred- to 1000-fold, characterized by C-reactive protein (CRP) and serum amyloid A (SAA) (Kushner and Mackiewicz, 1987).

The increases in plasma concentrations of the acute phase proteins following inflammatory stimulus largely results from increased rates of synthesis and secretion by hepatocytes (Baumann et al., 1984; Schultz and Arnold, 1990; Kushner and Mackiewicz, 1987). Although extrahepatic synthesis of some acute phase proteins has been demonstrated, that aspect of production plays only a minor role in elevating blood levels of these proteins (Kushner and Mackiewicz, 1987).

The acute phase proteins provide important protective functions, participating in the defense of the host organism against tissue damage and infections. C3 and CRP, for example, aid in the opsonization of bacteria, parasites, foreign particles, and immune complexes, and aid in their

removal by phagocytic cells. Fibrinogen plays an integral part in blood clotting and wound healing. Haptoglobin is a major component of the salvage mechanism to save iron when free hemoglobin is released into the blood stream. Many of the acute phase proteins are proteinase inhibitors: α_1-anti-trypsin inhibits leukocyte esterase, thereby preventing serious tissue damage caused by this proteinase during inflammation of the lung; α_1-anti-chymotrypsin, α_1-macroglobulin and α_2-macroglobulin are broad spectrum proteinase inhibitors (Schultz and Arnold, 1990; Fey and Gauldie,1990). It has been suggested that AAG has an immunoregulatory role (Schultz and Arnold, 1990; Cheresh et al., 1984). AAG at 2.0 mg/ml had immunosuppressive effects on mitogen induced blastogenesis of mouse spleen cells and at concentrations of 0.5 mg/ml AAG had an immunosuppressive effect on human lymphocytes from patients suffering from different ailments (Cheresh et al., 1984). At concentrations of 20 - 50 μg/ml AAG had an immunostimulatory effect on mitogenesis of human lymphocytes (Singh and Fudenberg, 1986). The role of AAG in the acute phase response remains under investigation.

C-reactive protein

Tillet and Francis (1930) described a cell wall polysaccharide fraction (Fraction C) from a strain of Pneumococcus. Fraction C caused a precipitate to form when it was added to sera of patients with febrile pneumococcal

infections, but not when added to sera of the same patients after recovery or when added to normal sera. The precipitation with Fraction C was observed with sera from patients with hemolytic streptococcal pharyngitis infections and some non-infectious diseases. The serum component which precipitated with Fraction C was named C-reactive protein. Since its discovery CRP has been regarded primarily as a useful clinical marker to monitor inflammation and the occurrence and recurrence of neoplastic growth (Fey and Gauldie, 1990; Schultz and Arnold, 1990).

CRP is a heat labile, non-immunoglobulin serum substance which requires calcium for precipitation of Fraction C. It is a β-globulin, and is classified in the super family of pentraxins, which are cyclic, non-glycosylated proteins constructed of five identical globular, non-covalently linked proteins. CRP consists of five 23 kDa subunits which combine to form a 117 kDa protein (Gotschlich, 1989). The CRP gene contains one intron and is present as a single copy on chromosome 1, near the area which contains the gene for another acute phase protein, serum amyloid P (Lei et al., 1985). The complete amino acid sequence of CRP is known, as is the nucleotide sequence from the cDNA (Whitehead et al., 1983). SAA and CRP are the only acute phase proteins which are not glycosylated (Schultz and Arnold, 1990).

CRP is a phylogenetically conserved protein, with sequence and structural homology present across several vertebrate

species (mammals, chicken, fish) as well as some invertebrates (Pepys and Baltz, 1983). For example, Limulus polyphemus, the horseshoe crab, constitutively produces large amounts of a CRP-like compound as a major component of its hemolymph.

Each subunit of CRP has a phosphocholine binding site which binds through Ca^{2+} to substrates bearing phosphocholine, a constituent of cell wall membranes (Schultz and Arnold, 1990). CRP has been shown to act as a nonspecific opsonic factor in inflammatory states, and receptor-mediated binding of CRP to polymorphonuclear (PMN) leukocytes, monocytes and lymphocytes has been demonstrated (Linke et al., 1991; Schultz and Arnold, 1990; Zahedi, et al., 1989). The interaction between CRP and PMNs appears to be particularly important. Inflammatory stimuli cause dramatically increased hepatic synthesis and secretion of CRP concomitantly with neutrophilia and migration of PMNs into damaged tissues at the site of inflammation Buchta et al., 1987). PMNs, monocytes, and macrophages within the inflamed tissue eliminate the damaged area and allow tissue repair to take place (Fey and Gauldie, 1990; Schultz and Arnold, 1990). Several studies examining the binding of CRP to phagocytic cells have shown that CRP binds to both PMNs and macrophages through specific, saturable binding to receptors with a k_d of 10^{-8}M to 10^{-7}M (Buchta et al., 1987; Zahedi et al., 1989; Ballou et al., 1989).

CRP has been shown to stimulate the classical pathway of complement and to inhibit the alternate pathway of complement

(Schultz and Arnold, 1990). CRP inhibited the formation of lung metastases and prolonged survival of mice bearing malignant fibrosarcomas (Schultz and Arnold, 1990, Zahedi et al., 1989), and protected against fatal streptococcus pneumonia infection (Yother et al., 1982).

CRP binds to chromatin or chromatin fragments in a Ca^{2+} dependent reaction and may mediate their solubilization (Robey et al., 1984; Robey et al, 1985). A primary role of aggregated CRP is to aid in opsonization, therefore CRP-chromatin complexes should be readily phagocytized. These observations have led to the proposal of an additional role for CRP as a scavenger and clearance factor for chromatin fragments released from cells damaged during an inflammatory process.

Serum amyloid A

Serum amyloid A was first identified approximately 15 years ago as the precursor of protein amyloid A (AA), the proteinaceous fibril material that is a major component of amyloid deposits in secondary reactive amyloidosis (Benson et al., 1975a; Linke et al., 1975; Anders et al., 1975). Amino acid sequence data confirmed that AA represented the amino-terminal 2/3 of SAA (Schultz and Arnold, 1990).

The concentration or SAA in plasma may increase as much as 1000-fold to levels of 1.0 mg/ml following inflammatory stimuli (Schultz and Arnold, 1990). SAA mRNA has been shown to increase as much as 2000 fold to some 20,000 copies per cell following endotoxin administration (Linke and Stolle,

1984), which demonstrated that the observed increase in serum level was due to an increase in message, and not to release of stored SAA (Linke et al., 1984; Schultz and Arnold, 1990). Although primarily synthesized in the liver, the SAA gene is expressed in extra-hepatic sites in the mouse in organs where amyloid is deposited (Schultz and Arnold, 1990). SAA has been isolated from cultures of interleukin (IL)-1 or phorbol 12-myristate 13-acetate (PMA) stimulated rabbit fibroblasts (Mitchell et al., 1991).

SAA is also a phylogenetically conserved protein. Human, murine, mink, and rabbit SAA all possess an invariant portion and are 50-70 % conserved in their primary amino acid sequence (Schultz and Arnold, 1990; Meek and Benditt, 1989; Yamamoto and Magita 1985; Bausserman et al., 1980; Parmelee et al., 1982). The human SAA genes are located on the short arm of chromosome 11, and have been localized to a 90 kb region (Sack et al., 1989). There are at least 3 genes for SAA, as suggested by the existence of two major and 4 minor isotypes in human serum. The major two isotypes vary only in a double substitution of valine for alanine at position 52 and 57 (Malmendier and Lontie, 1988). The DNA sequences of three recombinant clones which have been isolated and sequenced predict the two isotypes noted above, and a third, somewhat divergent isotype (Malmendier and Lontie, 1988). SAA is a cysteine-free single peptide of 11,685 daltons, comprised of 104 amino acids, which possesses the amphipathic structure of

an apolipoprotein (Malmendier and Lontie, 1988; Steinkasserer et al., 1990). Structurally, SAA is predicted (by the Chou-Fasman protein secondary structure algorithm) to consist of a short, positively charged amino terminus two amino acids long, followed by a central hydrophobic region which could span a cellular lipid bilayer (16 amino acid residues in length). The first 24 amino acids of SAA form an amphipathic α helix of about 7 turns. Amino acids 28 - 75 are predicted to form a series of 4 hydrophobic ß sheets and ß hairpin turns, followed by the remaining 29 amino acids which are predicted to form another α helix (Steinkasserer et al., 1990).

Northern blot analyses indicate that all members of the SAA multigene family are expressed during the acute phase process (Steinkasserer et al., 1990). The two major species, SAA_1 and SAA_2, along with their counterparts with the N-terminal arginine removed, make up almost all of the SAA found in the plasma (Malmendier and Lontie, 1988). SAA is almost entirely associated with high density lipoprotein $(HDL)_3$ in plasma as a 180 kDa complex (Turnell and Pepys, 1986); as much as 50% of the apolipoprotein associated with HDL during an acute phase reaction may be SAA (Bausserman et al., 1980; Clifton et al., 1985). Synthesis of SAA by the liver is regulated independently of Apolipoprotein A-1 (Apo A-1), and SAA is secreted from the liver before it is incorporated into HDL particles (Bausserman et al., 1988). Bausserman et al., (1988) showed that SAA was unable to displace Apo A-1 from HDL particles.

The half-life of SAA in plasma is approximately 90 minutes, while the half-life of Apo A-1 is approximately 10 hours. During an acute inflammation, the half-life of Apo A-1 decreases to approximately 3.5 hours, while the half-life of SAA remains unchanged (Kisilevsky, 1991), despite the increase of several orders of magnitude in concentration of SAA. This indicates that the body has an enormous capacity to clear SAA from the circulation, a capacity which is not saturated even when concentration of SAA reach as high as 500 μg/ml (Tape and Kisilevsky, 1990).

HDL particles containing SAA were catabolized faster than normal HDL particles in both murine (Clifton et al., 1985; Hoffman and Bendit, 1982) and monkey (Parks and Rudel, 1983; Clifton et al., 1985) models, which led to the hypothesis that SAA acts as a delivery signal for the presentation of HDL particles to damaged tissue repair systems (Meek et al., 1989; Kisilevsky, 1991). At sites of tissue injury large numbers of cells are destroyed, resulting in the release of many lipid, protein, and nucleic acid components. Free fatty acids released by lipase attack of triglycerides or phospholipids, would provoke a further more intense inflammatory response. Lipids freed from lysed cells would likely be scavenged in large quantities by inflammatory cells such as macrophages at the site of tissue destruction, generating foam cells, lipid-laden or cholesterol-laden macrophages. Locally, the cholesterol could be reutilized and made available to

proliferating cells in the area of damage, or it could be removed from the inflammatory cells and transported to the liver for repackaging and utilization or for excretion. Using the HDL reverse cholesterol transport mechanism to remove the cholesterol from lipid laden inflammatory cells, or to remove free lipids from the site of inflammation, would serve to reduce the inflammation, thereby dampening the inflammatory response and preventing the large scale destruction of healthy tissues surrounding the site of tissue damage.

Kisilevsky (1991) postulated that cytokines generated at the sight of inflammation induce the liver to produce large quantities of SAA, which then associate with HDL and serve to redirect HDL to lipid-laden cells such as foam cells, a site of excess cholesterol uptake and storage. The HDL particle would then transport cholesterol away from the macrophage.

SAA has specific wound healing and immunomodulatory activities. Mitchell et al., (1991) showed that SAA induced the synthesis of collagenase in rabbit synovial fibroblasts, and that this stimulation was reversed by the addition of anti-SAA to the culture. SAA concentrations as low as 0.1 μg/ml (10^{-8} M) inhibited the oxidative burst response of neutrophils observed when they were incubated with 10^{-7} M N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Linke et al., 1991). This FMLP stimulated oxidative burst response was restored by incubation of the SAA treated neutrophils with anti-SAA. SAA did not, however, inhibit the neutrophil oxi-

dative burst response to PMA, which activates protein kinase C and bypasses cytosolic signal transduction. This indicated that SAA specifically modulated the heterogeneous signal response to the FMLP-receptor (Linke et al., 1991).

SAA inhibited thrombin-induced gel-filtered platelet aggregation, but did not inhibit aggregation induced by collagen or adenosine diphosphate. The aggregation of platelet-rich plasma activated with thrombin was unaffected. At concentrations of 25 to 100 μg/ml SAA suppressed the increase in cytosolic Ca^{2+}, thromboxane generation, and suppressed serotonin release. SAA did not affect the clotting activities of thrombin (Zimlichman et al., 1990). These findings suggest that SAA may play a protective role in thromboembolic disease by suppressing thrombin-induced platelet aggregation.

As early as 1975 Benson and Aldo-Benson showed that SAA inhibited the secondary antibody response of murine spleen cells isolated from mice primed with sheep red blood cells (SRBCs). In 1979 the same investigators demonstrated that SAA inhibited the primary in-vitro immune response of murine spleen cells to SRBCs, and that this suppression was blocked with anti-AA; however, when spleen cells were stimulated with the T-independent antigen dinitrophenol (DNP)-ficoll, SAA had no suppressive activity. The observed suppression occurred in a dose-dependent manner, whether SAA rich serum or purified SAA was used. The anti-SRBC response was inhibited 83% by the

addition of 100 μg/ml (8μM) SAA and 70% by the addition of 10 μg/ml SAA. Even after 6 hours incubation, the SAA induced suppression of anti-SRBC response could be removed by washing the cells in saline, suggesting that the suppression was a cell surface associated phenomenon. SAA added during the first 24 hours of culture suppressed the response, but SAA added after the initial 24 hours had no effect. This implies that SAA has suppressive activity only during the recognition/differentiation phase of the response, affecting the differentiation of antigen binding cells into antibody producing cells, but not the proliferation of antibody producing cells. Since SAA was suppressive when added after the initial 3 hours, it did not simply block antigen binding, which normally occurs during the first 1-3 hours of culture (Benson et al., 1975; Benson and Aldo-Benson, 1979; Benson and Aldo-Benson, 1982; Aldo-Benson and Benson, 1982).

There is no evidence that SAA suppresses immune responsiveness through the formation, differentiation, or proliferation of T-suppressor cells (Aldo-Benson and Benson, 1982). SRBC-stimulated T-cells cultured in the presence of SAA had no inhibitory effect when added to fresh cultures of SRBC-stimulated lymphocytes. Fresh T-cells added to SAA-suppressed cultures partially restored immune function, as did addition of fresh macrophages, while complete function was restored only by addition of both T-cells and macrophages. These observations suggest that SAA may interfere with the

interaction between T-cells and macrophages (Benson and Aldo-Benson, 1982; Aldo-Benson and Benson, 1982) and the resultant production of stimulatory factors.

Peristeris et al., (1989) demonstrated that SAA added to mixed lymphocyte cultures to a final concentration of 30 μg/ml inhibited incorporation of ^{3}H-thymidine, but was not toxic to cells at levels of up to 300 μg/ml. SAA at 30 μg/ml inhibited both concanavalin A (Con A) and phytohemagglutinin (PHA) stimulated E-rosette formation (which characterizes activated T-cells) and PHA and Con A stimulated mitogenesis. SAA has been proposed as a pharmacological agent with potential use as an immunosuppressive agent (Linke et al., 1991).

Assay

The 1000-fold or higher increase in concentration of SAA during an inflammatory incident has created an interest in its clinical utility for monitoring inflammation in a variety of patient populations (Schultz and Arnold, 1990). SAA has been proposed for use as a marker for measuring inflammation in renal transplant rejection (Maury and Teppo, 1984; Hocke et al., 1989), and to serve as a useful assessment or measurement of inflammation due to pulmonary infection in cystic fibrosis patients (Marhaug et al., 1983). The disease activities of patients with rheumatoid arthritis, secondary reactive amyloidosis, neoplastic diseases (Benson and Cohen, 1979), myocardial infarction (Clifton et al., 1985), and trauma (Choukaife et al., 1989) can also be assessed in part by

measuring SAA levels in plasma. Autoantibodies to SAA have been observed in patients with systemic lupus erythematosus (SLE), and may be partially responsible for some of the sequelae or complications of this disease (Linke and Stolle, 1984). It has been suggested that the removal of SAA from circulation by autoantibodies in SLE and rheumatoid arthritis patients may be related to disease processes (Linke et al., 1991; Linke and Stolle, 1984; De Beer et al., 1982).

The clinical utility of measuring plasma or serum concentrations of SAA has been hampered by the lack of commercial antisera, standards, and well characterized assays (Chambers et al., 1991). The development and characterization of an appropriate assay is dependent on a reliable source of purified SAA to be used as a standard. Several protocols describing the purification of SAA from acute phase sera have been published. Most require several long ultracentrifugation steps to separate the HDL_3 fraction from acute phase sera, followed by sequential gel filtration under dissociating conditions (Bausserman et al., 1980; Raynes and McAdam, 1988; Steinmetz et al., 1989; Strachan et al., 1989). These procedures are time consuming, expensive, and generally result in low (15 - 30 %) recoveries of SAA (Raynes and McAdam, 1988).

The amphipathic nature of SAA led to the investigation of the use of hydrophobic interaction chromatography as a major step in its purification. Hydrophobic interaction chromatography media provides hydrophobic moieties which bind

the hydrophobic surface areas of proteins, such as the extremely hydrophobic N-terminal 11 amino acids of SAA (Husby et al., 1988). The bound protein is then eluted by reducing the ionic strength or the polarity of the eluant or by increasing its hydrophobicity.

Raynes and McAdam (1988) used hydrophobic interaction chromatography as the initial step in their purification of SAA. The procedure reported employed high concentrations of guanidine to elute the SAA from phenyl sepharose or octyl sepharose, followed by gel filtration in 3.0 M guanidine and ion exchange chromatography in 4.5 M urea.

A variety of assays for SAA have been developed, most of which require either chemical or thermal denaturation of the plasma or serum to ensure the complete dissociation of SAA from the HDL_3 fraction of lipoprotein (Marhaug et al., 1983; Sipe et al., 1989). The difficulties associated with the development of assays which require a denaturation step have been noted by several investigators, (Marhaug et al., 1983; Pepys and Baltz, 1983; Benditt et al., 1988; Sipe et al., 1989). Denaturation by heat, acid, or alkali appears to lead to considerable variability in the reproducibility of the assays (Sipe et al., 1989).

Several antibody sandwich enzyme linked immunosorbant assays (ELISA)s have been described, employing rabbit (Dubois and Malmendier, 1988), or rat antisera against SAA (Zuckerman and Suprenant, 1986), rabbit antisera against AA purified from an

amyloidotic human kidney (Yamada et al., 1989), or rabbit antisera against discrete peptides of SAA (Saile et.al., 1989). The problems associated with clinical assays which employ polyvalent antisera, (i.e., the lack of reproducibility of antisera titers from one lot to the next, and the substantial cross-reactivity which may be present) permit only limited usefulness in the clinical laboratory setting. The high degree of specificity, reproducibility and sensitivity associated with sandwich ELISAs utilizing monoclonal antibodies, however, make this type of test ideal to adapt for use in a clinical laboratory.

Development of an adequate supply of specific, high affinity, monoclonal antibodies is a prerequisite for development of a monoclonal antibody sandwich ELISA assay (Harlow and Lane, 1988). Monoclonal antibodies with affinity for separate, non-overlapping epitopes of the antigen to be detected must be available. The affinity and specificity of the antibodies is dependent on the species of animal from which the spleen cells are obtained, as well as the procedures employed during immunization, fusion and screening techniques, etc. As noted previously, SAA is a phylogenetically highly conserved molecule, with limited molecular differences observed between species (Schultz and Arnold, 1990; Meek and Benditt, 1989; Yamamoto and Magita 1985; Bausserman et al., 1980; Parmalee et al., 1982). The generation of monoclonal antibodies to non-overlapping epitopes of a protein requires

that the immunogenic epitopes be "foreign" to the animal used to generate the monoclonal antibody. Rats do not produce detectable amounts of SAA (Kushner and Mackiewicz, 1987) and are therefore an ideal source of spleen cells for the production of high affinity monoclonal antibodies to separate, non-overlapping epitopes of SAA.

Cystic fibrosis

Cystic fibrosis (CF) patients have a very high incidence of lung infections which are most commonly due to a mucoid variant of Pseudomonas aeruginosa (Gilligan, 1991). This chronic infection is associated with slowly progressive irreversible damage to lung tissue, which ultimately leads to the death of the majority of CF patients. Treatment reduces but rarely eradicates the infection. Normally, protection against lung infections is provided by nonspecific and specific defense mechanisms. Airway reflexes, mucociliary clearance and the epithelial barrier provide the initial, non-specific defenses (Pederson et al., 1989). The mucus lining of the airways contains antibacterial proteins such as lysozyme, complement, lactoferrin, transferrin, and such phagocytic cells as alveolar macrophages and neutrophils. No primary defect of these mechanisms has been documented in CF patients (Moss, 1983; Pedersen et al., 1989).

The specific types of defense mechanisms include the cellular and humoral immune system. These systems provide targeted lymphocytes and antibodies which recognize specific

antigens and lead to killing of bacteria by cytotoxic reactions or opsonization and phagocytosis. Evidence is mounting that antibodies, through the formation of immune complexes and consequent activation of complement, contribute to the pathogenesis of Pseudomonas aeruginosa infections (Schiotz, 1989). When bacteria enter the lung, antibody molecules bind, immune complexes form, and these complexes then are eventually phagocytosed. If there is an excess of antibody available, the complexes may become large enough to precipitate, and elicit inflammation. In an equilibrium situation, i.e., with equivalent amounts of antigen and antibody, large immune complexes may form and initiate inflammation via the complement system (Schiotz, 1989). The concentrations of immune complexes have been measured in cystic fibrosis patients and found to correlate with decreasing lung function, disease chronicity, and low National Institutes of Health (NIH) scores in these patients (Disis et al., 1986; Schiotz, 1989).

The immune system in the CF patient is obviously somehow inefficient in destroying or preventing infection with P. aeruginosa, as the infection becomes chronic and eventually life-threatening. The use of steroidal anti-inflammatory therapy to control the excessive inflammatory response in this patient group has gained some support in recent years. Auerbach (1989) showed in a four year study that patients who received alternate day prednisone therapy required fewer hospitalizations and had significant advantages over the

placebo group in terms of increased growth rate (as measured by height and weight), pulmonary function, erythrocyte sedimentation rate, and serum IgG levels. Corticosteroid therapy in CF patients is currently used primarily for the treatment of intractable asthma, wheezing and chest tightness which are unresponsive to aerosolized bronchodilators, and is the treatment of choice for allergic bronchopulmonary aspergillosis (Gilligan, 1991).

Determining when antibiotic therapy is required to control a P. aeruginosa associated pulmonary exacerbation presents a significant challenge to the clinician. Discerning colonization from active infection may be difficult or impossible on the basis of quantitative sputum cultures. Patient assessments based on chest X-rays, weight loss, anorexia, erythrocyte sedimentation rate, leukocyte count and pulmonary function tests, while useful, may not provide results which accurately determine patient status.

Determination of CRP concentration provides a rapid, reliable measure of inflammation in patients with a variety of diseases including CF. Glass, et al., (1987) showed that CRP levels above 10 mg/liter were associated with pulmonary exacerbation in CF patients, and the return of CRP levels to normal range coincided with clinical resolution of the exacerbation. These observations have led to the widespread use of CRP measurements as an index of acute or impending

pulmonary exacerbation in CF patients, and as a means to monitor the efficacy of antibiotic therapy.

SAA was shown to be more sensitive than CRP or orosomucoid as an indicator of lung inflammation in CF patients (Marhaug et al., 1983) and its elevated levels correlated with the presence of pathogens in the sputum. A decrease in the concentration of SAA during antibacterial therapy was shown to be a good indicator of efficacious therapy, though frequently the concentration of SAA never returned to "normal" levels in these patients.

Production of acute phase proteins

IL-1α, IL-1β, IL-6, tumor necrosis factor (TNF), and leukemia inhibitory factor (LIF) are the major cytokines involved in the regulation of the synthesis of acute phase proteins (Schultz and Arnold, 1990; Raynes et al., 1991). Recently it has been reported that the activities, cellular receptors and binding affinities of IL-1α and IL-1β are not identical, as had been thought for some time. IL-1α and IL-1β have only a 26% homologous amino acid sequence (Endres et al., 1987), and it was recently demonstrated that the two IL-1 genes have very little in common (Mora et al., 1990).

The receptor expression and cellular distribution for IL-1α and IL-1β also differ significantly (Scapigliati et al., 1989). Scapigliati demonstrated that unlabelled IL-1β displaced both radiolabelled IL-1α and IL-1β from RAJI cells, while unlabelled IL-1α displaced radiolabelled IL-1α, but was

unable to displace radiolabelled IL-1β. The receptor which predominates on T cells preferentially binds IL-1α and has a molecular weight of 80 kDa. The B cell receptor, which binds only IL-1β, has a molecular weight of 68 kDa. Ghiara et al. (1990), recently demonstrated that the human hepatoma cell line HepG2 possesses both IL-1 receptors, i.e., the receptor which predominates on T-cells (average 417 per HepG2 cell), as well as the receptor which predominates on B-cells (average 1400 per HepG2 cell). HepG2 cells thus possess two structurally different IL-1 receptors with distinct binding properties for IL-1α and IL-1β (Ghiara et al., 1990).

Castell et al. (1988), demonstrated that primary cultures of human hepatocytes obtained from normal liver during elective cholecystectomy responded to IL-6 by producing SAA and CRP, and that the production of SAA was enhanced by the presence of the corticosteroid dexamethasone while the production of CRP was unaffected. Moshage et al., (1988) found that primary cultures of human hepatocytes responded to various combinations of recombinant human (rh)IL-1 and rhIL-6 by producing SAA and CRP. Several human hepatoma cell lines have been shown to be capable of producing most of the acute phase proteins when exposed to the appropriate stimulatory cytokine "soups" (Ganapathi et al., 1991; Mackiewicz et al., 1991) or to conditioned media obtained from monocytes stimulated with lipopolysaccharide (LPS) (Baumann et al., 1984; Ganapathi et al., 1988) or with phytohemagglutinin (Huang, et al., 1990).

HuH-7 human hepatoma cells produce SAA when stimulated with IL-1α, IL-1β, or IL-6 (Raynes et al., 1991). In contrast, neither Il-1 nor IL-6 alone was able to induce the production of SAA or CRP from Hep3B cells (Ganapathi et al., 1991); however, the combination of IL-1 and IL-6 did induce production of both SAA and CRP in that cell line.

HepG2 cells are the most morphologically "normal" of the human hepatoma cell lines, and appear relatively well differentiated (Bouma et al., 1989). They do not produce measurable concentrations of the acute phase proteins unless they are stimulated with inflammatory cytokines (Bouma et al., 1989; Richards et al., 1992). In addition, HepG2 cells are free of hepatitis B virus (Hay et al., 1988), and thus do not require extensive containment facilities for infection control.

Glucocorticoids have variable effects on the production of acute phase reactants from hepatoma cell lines induced with monocyte conditioned media (MOCM) (obtained by stimulating monocytes from peripheral blood with E. coli LPS or with a suspension of killed P. aeruginosa) or with cytokine "soups". Some cells demonstrate significantly increased production of SAA compared to CRP when dexamethasone is added to the incubation mixture (Castell et al., 1988), while others show equivalent enhancement of production of both SAA and CRP (Steel and Whitehead, 1991).

SPECIFIC AIMS:

This dissertation contains four manuscripts which report the results of studies undertaken to purify SAA from serum, to develop an assay for measuring SAA concentrations in serum or in tissue culture fluid, to assess the clinical utility of measuring SAA concentrations in the sera of patients undergoing acute inflammatory processes, and to examine the production of SAA and CRP in an in vitro system. The in vitro system was developed in an attempt to address an observed disparate production of SAA compared to CRP in corticosteroid treated CF patients, and pursue a tenable explanation for the observed disparate production. The specific aims of each of the four areas addressed in these studies are listed under the title of the appropriate manuscript.

Manuscript #1:

Use of Ethanol Eluted Hydrophobic Interaction Chromatography in the Purification of Serum Amyloid A.

Specific Aims:

1.To purify SAA to homogeneity and obtain sequence data to prove that the protein under study was SAA.

2. To develop a reliable, easily obtainable source of SAA to be used as a standard for development of an assay for measuring SAA in the sera of patients and in tissue culture fluid.

3. To purify SAA in the absence of guanidine and/or high concentrations of urea so that the SAA obtained might be

used for in vitro experiments without concern for potential biological activity of those reagents.

Manuscript #2.

A Monoclonal Antibody Sandwich Immunoassay for Serum Amyloid A (SAA) Protein.

Specific Aims:

1. To develop a specific, sensitive, and reproducible assay employing two monoclonal antibodies to SAA which were available in the laboratory. The purpose of the assay was to determine SAA concentration in the sera of patients undergoing acute phase processes.

2. To characterize the assay and determine its suitability for use in a clinical laboratory setting by measuring SAA concentrations in the sera of a group of patients and correlating SAA levels with CRP levels in those sera.

Manuscript #3.

Serum Amyloid A is More Sensitive than C-reactive Protein as an Indicator of Lung Inflammation in Both Corticosteroid Treated and Non-Corticosteroid Treated Cystic Fibrosis Patients.

Specific Aims:

1. To determine SAA concentrations in the sera of CF patients and correlate the SAA concentrations with CRP concentrations in the same sera.

2. To correlate both CRP and SAA concentrations with pulmonary function as determined by forced expiratory volume in 1 second (FEV1), peak expiratory flow rate (PEFR), and forced vital capacity (FVC).

3. To assess the effect of systemic corticosteroid anti-inflammatory therapy on levels of CRP and SAA in cystic fibrosis patients.

4. To determine whether measuring SAA levels provides any new information (not provided by measuring CRP levels) which would be of value to the clinician and aid in the assessment of the efficacy of therapy for these patients.

Manuscript #4.

Production of Serum Amyloid A and C-reactive Protein by HepG2 Cells Stimulated with Combinations of Cytokines or Monocyte Conditioned Media: The Effects of Prednisolone.

Specific Aims:

1. To determine the effects of the corticosteroid prednisolone on the production of IL-1α and IL-1β by monocytes stimulated with either LPS from E. coli or with a suspension of killed P. aeruginosa, the primary agent for most lung infections of cystic fibrosis patients.

2. To assay the bioactivity of the monocyte conditioned media obtained as described in specific aim #1 above by measuring the production of SAA and CRP by HepG2 cells stimulated with the conditioned media, and determine whether the bioactivity was due to IL-1α, IL-1β, or both.

3. To determine the effect of prednisolone on the bioactivity of conditioned media obtained from monocytes stimulated with E. coli LPS in the absence of prednisolone.

4. To measure the production of SAA and CRP by HepG2 cells stimulated with different combinations and concentrations of rhIL-1α, rhIL-1β, rhIL-6, and rhTNF-α in the absence or in the presence of several concentrations of prednisolone.

RESULTS AND DISCUSSION:

Manuscript #1 A two step scheme for the purification of SAA from acute phase sera is described. In the first step hydrophobic (lipidic) components of acute phase sera were adsorbed to the hydrophobic interaction chromatography media octyl sepharose. After washing off the nonbound material, the bound material was eluted with increasing concentrations of ethanol. The SAA containing fractions were concentrated via ultrafiltration, and dialyzed against 10% formic acid. The SAA concentrate in formic acid was then rechromatographed on a gel filtration column using 10% formic acid as eluant. After dialyzing overnight in phosphate buffered saline containing .01% sodium dodecyl sulfate (SDS) the SAA positive fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and submitted for 15 cycles of N-terminal amino acid sequence analysis. The results of the amino acid

sequence analysis, R S F F S F L G E A F D G A R, were identical with the published sequence for SAA.

The overall recovery of SAA using this purification scheme was 56% of a >92% pure compound. SAA purified by this method offers a reliable source of purified standard for the development of an SAA assay. The SAA positive fractions isolated from the first step of the purification are also suitable for use as a standard.

This method is the first published purification of SAA accomplished without the use of either ultracentrifugation or high concentrations of expensive and potentially biologically active guanidine and/or urea. SAA obtained by this method could be made available in 3 days from the time the acute phase sera were collected. The chromatography was accomplished using an automated fast protein liquid chromatography (FPLC) system which allows for easy scale up. This protocol represents a viable method to procure a standard for large-scale utilization of an assay to measure SAA concentrations in sera. As the purification involves no potentially biologically active guanidine or urea the dialysis procedures required to obtain a sufficiently pure sample to test in biological systems are significantly simplified.

Manuscript #2 The development and clinical utility of an ELISA for SAA is described. This assay utilizes two monoclonal antibodies which bind separate, nonoverlapping epitopes of SAA; one antibody to capture SAA from diluted

serum samples, and the other, conjugated to alkaline phosphatase, to detect captured SAA. The assay was found to be sensitive, reliable, reproducible, and could be readily adapted for use in a clinical laboratory.

Both monoclonal antibodies (designated 5G6 and 6B10) used for the assay bind human SAA on Western blots, while only one (5G6) binds murine SAA. This observation showed that the two antibodies bind separate epitopes of SAA, with the 5G6 antibody binding an epitope of SAA which is well conserved between human and murine SAA, and the 6B10 antibody binding to an epitope which is not so well conserved. The methodology of the assay requires that the two antibodies bind different epitopes of SAA. The primary antibody, with which the ELISA plate is coated, captures SAA in such a fashion that the epitope to which the secondary, labeled antibody binds is exposed (Harlow and Lane, 1988).

The sensitivity of the assay is approximately 2.5 ng/ml of SAA in tissue culture fluid or PBS/0.01% Tween 20. The assay is linear for SAA levels from 2.5 to 250 ng/ml. The coefficient of variation for the standard curve averaged 6.5%; the interassay coefficient of variation averaged 7.8% while the intraassay coefficient of variation averaged 4.9%. The reported assay has advantages over the assays of Taktak and Lee al., (1991) and Sipe et al., (1991) in terms of increased sensitivity and the design of the assay, in that it requires no denaturation of the sample.

SAA in serum samples was found to be stable when stored for 30 days at 4°C, and for six months at -20°C. Recovery experiments showed that purified SAA added to SAA negative sera was fully accounted for (average recovery 98.3%) when assayed. The Pearson's correlation coefficient comparing SAA and CRP concentrations in serum samples from 180 patients was 0.69. This indicated that there was a strong correlation between SAA and CRP.

An inflammatory process is best followed by measuring one of the acute phase reactants, and SAA is the probably the best of the acute phase reactants to measure for this purpose. The four-fold greater incremental range of SAA compared to CRP in acute phase serum makes it a much more sensitive indicator of inflammation than CRP, as has been demonstrated by several investigators (Marhaug et al., 1983; Maury and Teppo, 1984; Whicher et al., 1985; Janssen et al., 1986; Chambers et al., 1987; Syrjanen et al., 1989). The assay described affords a means of measuring SAA concentrations in the sera of patients undergoing an acute inflammatory process, and is readily adaptable for use in the clinical laboratory.

Manuscript #3 The clinical utility of measuring SAA levels in serum of CF patients using the assay described in manuscript #2 is evaluated. SAA was found to be more sensitive than CRP as an indicator of pulmonary inflammation consequent to lung infections with <u>P. aeruginosa</u>, the principle cause of morbidity and mortality of cystic fibrosis

patients (Gilligan, 1991). SAA and CRP levels were determined in 830 sera from 155 CF fibrosis patients. The Pearson's correlation coefficient comparing SAA and CRP levels in these samples was 0.73 when all 830 samples were compared. There were 43 samples which did not fit into the expected pattern when the data points were plotted, i.e., the SAA levels were significantly elevated but the CRP levels were within normal range or were only slightly elevated. Chart reviews indicated that 35 of these 43 samples were obtained from CF patients treated with systemic anti-inflammatory corticosteroids (CFS). Accordingly, the data were reanalyzed excluding samples which were obtained from CFS patients. The correlation coefficient comparing SAA and CRP concentrations for the 698 samples from CF patients not treated with corticosteroids (CFNS) increased to 0.80, which indicated a strong correlation between CRP and SAA levels in this patient population. The correlation coefficient comparing SAA and CRP levels from the remaining 132 samples obtained from CFS patients was only 0.35.

During the course of these investigations 40 CFNS and nine CFS patients admitted to the hospital for seven or more days to receive intensive antibiotic therapy for lung infections due to P. aeruginosa. SAA and CRP levels were correlated with pulmonary function tests on admission and again on discharge for for each of these patients. The admission values for the pulmonary function tests were not statistically different when compared for the two groups, which indicated that the two

groups were admitted to the hospital with equivalent loss of pulmonary function. During their hospitalization both groups showed equivalent improvement in pulmonary functions, and were discharged with equivalent pulmonary function testing results, which implied that the hospitalization and antibiotic therapy was approximately equally effective for each group. During hospitalization forced vital capacity (FVC) of CFS patients improved from an average of 61% to 79% of expected values, while for CFNS patients FVC increased from 54% to 77% of expected values. Forced expiratory volume in 1 second and peak expiratory flow rate also showed equivalent increases for each group.

SAA levels at admission or at discharge were not statistically different between the two groups, which demonstrated equivalent declines toward normal levels during treatment. The notable variance between the two groups was that the average CRP concentration in CFS patients on admission was only 18.3 μg/ml, approximately 1/3 the concentration in CFNS patients (55.0 μg/ml). It is of interest to note that three of the nine CFS patients admitted to the hospital with P. aeruginosa lung infections and resultant loss of pulmonary function were admitted with CRP levels within normal limits, while all 9 had elevated SAA levels on admission. Of the 40 CFNS patients hospitalized during the course of this study, 39 had elevated CRP levels on admission and all had elevated SAA levels. Both SAA and CRP

levels in each group declined toward normal during the course of inpatient therapy.

In three CF patients hospitalized for treatment of P. aeruginosa lung infections, SAA and CRP levels showed an aberrant pattern. After an initial decline in both SAA and CRP levels, which coincided with the initiation of intensive antibiotic therapy, SAA concentrations increased while CRP concentrations remained the same or continued to decline slowly. In each case, evolving antimicrobial resistance of the P. aeruginosa forced a change in the antibiotic therapy utilized to control the infection. The increases in SAA concentration predated the physician's decision to adjust antibiotic therapy by 1 to 3 days. This suggests that SAA may be predictive of antibiotic therapy efficacy, and that a rising SAA level may indicate a need to reassess the antimicrobial susceptibility of the pathogen being treated.

SAA levels appear to have significantly greater sensitivity than CRP levels for the measurement of inflammation due to lung infections in CF patients. In all 49 CF patients hospitalized during the course of this study, elevated SAA levels were apparent on admission, which correlated with significantly decreased pulmonary function due to P. aeruginosa lung infections. Three of nine CFS patients and one of 40 CFNS patients were hospitalized with CRP concentrations within normal limits, yet had sufficient other

clinically significant symptoms of lung infection to warrant hospitalization for intensive antibiotic therapy.

Manuscript #4 The development of an in vitro model system to study the disparate production of SAA compared to CRP in CFS patients is reported. Prednisolone at concentrations ranging from 0.1 to 10.0 μM significantly inhibited the production of both IL-1α and IL-1β by monocytes stimulated with either E. coli LPS or with a suspension of killed P. aeruginosa. The bioactivity of the monocyte conditioned media (MOCM), as measured by induction of SAA and CRP production by HepG2 cells, mirrored the concentrations of IL-1α and IL-1β. When the concentrations of the IL-1α and IL-1β were highest, the bioactivity of the MOCM was highest, and when the concentrations of IL-1α and IL-1β were low, so was the bioactivity. It thus appears that corticosteroids may decrease the "signal" which arises from activated monocytes/macrophages, the "signal" which drives the production of the acute phase reactants by hepatocytes. This finding confirmed data from previous observations (Ghezzi and Sipe, 1988; Ganapathi et al., 1988) and offers a direct measurement of the effects of corticosteroids on the production of the IL-1α and IL-1β by monocytes. Monocyte production of IL-1α and IL-1β in response to stimulation with E. coli LPS, or with a suspension of killed P. aeruginosa, was unaffected by SAA. SAA was added to cultured quiescent monocytes either simultaneously with the stimulant or six

hours prior to stimulation. SAA was added to obtain final concentrations ranging from 10 to 400 μg/ml in either (1) a purified form (>90% SAA) without associated lipid or HDL fraction, (2) a partially purified form (35% SAA), in complex with HDL and containing other apolipoproteins or (3) as crossmatch compatible SAA rich serum. Monocytes were not activated by the addition of SAA, as no measurable production of IL-1α or IL-1β occurred. The bioactivity of the MOCM obtained from SAA treated monocytes as measured by the production of CRP by HepG2 cells was equivalent to MOCM from monocytes which were not treated with SAA (Data not shown). These observations suggest that the suppressive activity of SAA on lymphocytes, platelets, and neutrophils (Benson et al., 1975; Benson and Aldo-Benson, 1979; Benson and Aldo-Benson, 1982; Peristerous et al., 1989; Zimlichman et al., 1990; Linke et al., 1991) does not extend to monocytes.

Neutralization studies, wherein the activity of either IL-1α or IL-1β in MOCM was neutralized with specific monoclonal antibodies, showed that neutralization of either IL-1 reduced the bioactivity of the MOCM. However, neither IL-1 was wholly responsible for the bioactivity associated with the MOCM. When both IL-1α and IL-1β were neutralized all the bioactivity of the MOCM was removed, which showed that either IL-1α or IL-1β was required for bioactivity of the MOCM. When prednisolone was added to a final concentration of 1.0 μM to MOCM prepared in the absence of prednisolone, the ability of

the MOCM to elicit the production of SAA from HepG2 cells was increased five- to six-fold, while the production of CRP was unchanged.

Media containing various combinations and concentrations of rhIL-1α, rhIL-1β, rhIL-6, and rhTNF-α were prepared in the absence or in the presence of prednisolone at several concentrations; these cytokine "soups" were then used to induce HepG2 cells to produce SAA and CRP. HepG2 cells required the presence of both rhIL-6 and rhIL-1 (either rhIL1-α or rhIL-1β) for induction of SAA and CRP production. Hep3B hepatoma cells were recently reported to have the same requirements as HepG2 cells (i.e., both rhIL-6 and rhIL-1) for the induction of SAA and CRP (Ganapathi et al., 1991). In contrast, Castell et al. (1988) showed that rhIL-6 was sufficient to elicit production of SAA and CRP from primary cultures of human hepatocytes, while Ganapathi et al., (1988) and Raynes et al., (1991) showed that rhIL-1 was sufficient to induce production of SAA and CRP from some hepatoma cell lines. Checkerboard titrations of different concentrations of rhIL-1α or rhIL-1β and rhIL-6 showed that maximal activity was reached with 25 ng/ml rhIL-1α or rhIL-1β and 5 ng/ml rhIL-6.

rhIL-1β was approximately five-fold more potent than rhIL-1α for the induction of SAA production by HepG2 cells. rhIL-1α and rhIL-1β had equivalent activity for the induction of CRP production, each capable of inducing approximately 80% of maximum activity at 30 ng/ml in the presence of 2.5 ng/ml

rhIL-6. rhIL-1β was considerably more potent than rhIL-1α for the induction of SAA production by HepG2 cells. rhIL-1α at a concentration of 30 ng/ml induced about 25% of maximum production of SAA in the absence of rhIL-1β, while rhIL-1β at a concentration of 30 ng/ml induced 100% of maximum SAA production in the absence of rhIL-1α. The differential production of SAA and CRP induced by rhIL-1α and rhIL-1β supports the findings of Ghiara et al.,(1991) and Scapigliati et al., (1989) who described two separate IL-1 receptors with different affinities for IL-1α and IL-1β.

Prednisolone significantly enhanced the production of SAA by HepG2 cells stimulated with either rhIL-1 in the presence of rhIL-6, but had no effect on the production of CRP. At 0.1 μM, prednisolone caused a three-fold increase in the production of SAA. When prednisolone was added to a final concentration of 1.0 μM, SAA production was increased by five-fold. Castell et al., (1988) described similar findings wherein 0.1 μM dexamethasone caused a six-fold increase in the production of SAA from IL-6 stimulated primary cultures of human hepatocytes, but had no effect on CRP production.

Corticosteroids are intimately involved in the regulation of production of the acute phase reactants. The production of the inflammatory cytokines IL-1α and IL-1β from LPS stimulated monocytes was inhibited by more than 50% by 1.0 μM prednisolone, and the bioactivity of the MOCM produced mirrored the concentration of IL-1α and IL-1β.

Corticosteroids acted directly on HepG2 cells stimulated with rhIL-1 and rhIL-6 to enhance the production of SAA, but not CRP. These observations offer a tenable explanation for the disparate production of SAA compared to CRP observed in CF patients receiving corticosteroid therapy.

SUMMARY

The measurement of CRP levels in the sera of CF patients provides a quantifiable value associated with the degree of inflammation that results from lung infections, and may aid in the diagnosis of such infections (Glass et al., 1987). Measurement of CRP concentrations provide a means to assess the efficacy of the care received, i.e., to determine whether the patient is improving or growing worse. Serum amyloid A, because of its higher serum concentrations and shorter half-life, provides a more dynamic and sensitive measure of inflammation than CRP. CRP levels appear to be unreliable as indicators of inflammation in corticosteroid treated CF patients, while SAA levels maintain their validity despite corticosteroid treatment. The validity of determining SAA level as a measure of inflammation in CF patients is shown by the data presented within this dissertation, as is the development of an assay appropriate for measuring SAA levels. There is no reason to suspect that measuring SAA levels in patients with other inflammatory processes occurring, especially those who frequently receive corticosteroid

therapy, i.e., transplant patients, rheumatoid arthritis patients, etc., would not be of equal value.

CONCLUSIONS:

Conclusions reached from experiments reported in each manuscript are summarized as follows.

Manuscript #1.

SAA was purified to homogeneity via hydrophobic interaction chromatography followed by gel filtration chromatography, avoiding the use of guanidine and/or urea.

Manuscript #2.

A two monoclonal antibody sandwich ELISA which could be readily adapted for use in a clinical laboratory was developed and characterized. The correlation coefficient comparing SAA and CRP concentrations ($r=0.69$) showed a strong correlation.

Manuscript #3.

SAA was more sensitive than CRP as an indicator of pulmonary inflammation in CF patients. In CFS patients SAA appeared to be a much more sensitive and reliable indicator of pulmonary inflammation than CRP.

Manuscript #4.

Prednisolone decreased the production of IL-1α and IL-1β by monocytes stimulated with LPS from E. coli, and the bioactivity of the MOCM produced in the presence of

prednisolone mirrored the concentrations of the IL-1α and IL-1β. Prednisolone enhanced the production of SAA by HepG2 cells, but had no effect on the production of CRP. HepG2 cells required both IL-1 and IL-6 to produce SAA and CRP. IL-1β was more potent than IL-1α for the induction of SAA by HepG2 cells, however the two IL-1s had equivalent activity for the production of CRP by HepG2 cells. These observations offer a tenable explanation for the disparate production of SAA compared to CRP observed in CFS patients.

SPECULATION ON THE ROLE OF SAA

The purpose of the acute phase response is to aid in the clearance of bacteria and other agents that initiate inflammation while preventing autolytic degradation of healthy tissue during an inflammatory response. Although the scope and modes of action of each of the acute phase proteins has not been fully delineated, important roles have been demonstrated for some. CRP aids in the opsonization of bacteria, and in the clearance of chromatin fragments released from lysed cells. Many of the acute phase proteins are protease inhibitors (α-1 antitrypsin, α-1 antichymotrypsin, etc.) which prevent large-scale destruction of healthy tissues. Several important activities, based on scientific observations, have been proposed for SAA. SAA has been shown to inhibit the immune response of lymphocytes to T-dependent

antigens, thrombin-induced platelet aggregation, and the oxidative burst response of neutrophils, as well as the proliferation of lymphoyctes in mixed lymphocyte cultures. Each of these findings suggest that SAA may dampen the immune response, perhaps allowing macrophages and neutrophils sufficient time to clear foreign materials and cellular debris, thus preventing an unnecessary activation of the immune response. Recent findings suggest an additional role for SAA in the removal of free fatty acids generated when damaged or infected cells are phagocytosed. This offers an additional mechanism by which SAA may dampen the immune response, by preventing free fatty acid activation of the immune system.

The role which SAA plays in the acute phase response and in the immune system in general will be further defined as investigations continue.

Title: Use of Ethanol Eluted Hydrophobic Interaction Chromatography in the Purification of Serum Amyloid A

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ABSTRACT: A two-step procedure for the purification of the acute phase reactant serum amyloid A from serum is described. A hydrophobic interaction chromatography media, Octyl Sepharose CL4B, eluted with increasing concentrations of ethanol was used as the first step in the purification. The concentrate from this step was applied to a gel filtration column of Sephacryl S-200 and eluted with 10% formic acid. The overall recovery of purified serum amyloid A from serum was 56%. This represents the first time that serum amyloid A has been purified without the use of high concentrations of guanidine or urea. The method presented could easily be scaled up to allow the purification of large quantities of serum amyloid A or readily adapted to the purification of other serum apolipoproteins.

INTRODUCTION:

Serum amyloid A (SAA) is an acute phase reactant produced primarily in the liver in response to an inflammatory process which may be infectious or non-infectious in nature. The hepatic production of SAA is presumably mediated by the activities of interleukin-1α, interleukin-1ß, interleukin-6, and tumor necrosis factor-α (Andus et al., 1991; Schultz and Arnold, 1990; Moshage et al., 1988). After synthesis in and secretion from the liver SAA associates with the HDL (high density lipoprotein) fraction of serum lipids where it may make up as much as 50% of the total apolipoprotein present in the HDL (Sipe et al., 1985). SAA serum concentrations approaching one mg/ml (1000 times the normal physiological concentration) have been reported in patients following trauma or infections (Sipe et al., 1985; Choukaife et al, 1989).

Serum amyloid A is a sensitive, accurate indicator of acute infection or tissue damage. Highest levels have been found in patients with acute myocardial infarction, followed by traumatic events, arthritis, viruses, bacterial infections and neoplasms (Schultz and Arnold, 1990). SAA proved to be more sensitive than C-reactive protein in detecting renal allograft rejection (Maury, 1985), and to be a very sensitive indicator of bacterial infection of the lung (Marhaug et al., 1983). These observations have led to an increased interest in the role that SAA may play in the acute phase response, where it has been proposed to be important in tissue repair and to

function as a recognition unit for HDL (Moshage et al, 1988). In order to further investigate these observations a dependable source of highly purified SAA was required.

Purification of SAA has generally been accomplished by first isolating the HDL_3 fraction from acute phase serum via several long ultracentrifugation steps, followed by delipidation and sequential gel filtration under dissociating conditions (Bausserman et al., 1980; Raynes and McAdam, 1988; Steinmetz et al., 1989; Strachan et al., 1989). These procedures are time consuming, expensive, and generally result in low (15 - 30 %) recoveries of SAA (Raynes and McAdam, 1988).

The amphipathic nature of SAA has led to the investigation of the use of hydrophobic interaction chromatography as a major step in the purification of this apolipoprotein. Hydrophobic interaction chromatography media provides hydrophobic moieties which bind the hydrophobic surface areas of proteins, such as the extremely hydrophobic N-terminal 11 amino acids of SAA (Husby et al., 1988). The protein can then be eluted by reducing the ionic strength or the polarity of the eluant or by increasing its hydrophobicity.

Raynes and McAdam (1988) recently reported the novel use of hydrophobic interaction chromatography as the initial step in the purification of SAA. SAA was eluted from either phenyl sepharose or octyl sepharose by increasing the concentration of guanidine in a linear gradient from zero to 4.0 M, in 30%

ethanediol, 10mM NaOH, followed by gel filtration in 3.0 M guanidine and ion exchange chromatography in 4.5 M urea.

This paper describes a two step purification procedure in which the SAA was eluted from octyl sepharose by increasing concentrations of ethanol, followed by gel filtration eluted with 10% formic acid, circumventing the use of guanidine and urea.

MATERIALS AND METHODS:

Serum Samples: The source of SAA was human serum samples on which C-reactive protein concentrations had been determined. These samples were made available from the Clinical Laboratory of the University of Nebraska Medical Center. Sera which had a C-reactive protein concentration greater than 5mg/dl were pooled and stored at 4° until used for SAA purification.

Chromatography: All chromatography was performed in the Monoclonal Antibody Core Facility at the University of Nebraska Medical Center utilizing a Pharmacia FPLC System with a Pharmacia-LKB LCC500 Plus controller and a Pharmacia UV1 monitor (Pharmacia, Piscataway, NJ).

Hydrophobic Interaction Chromatography: Twenty ml of pooled serum was diluted with 10 ml 50mM Tris-HCl, 10mM NaCl, pH 7.6 (Tris-NaCl buffer). This material was adsorbed onto a 1.0 cm X 9.0 cm column of Octyl Sepharose CL-4B (Pharmacia,

Piscataway, NJ) which had been preequilibrated with 5 column volumes of Tris-NaCl buffer. The column was washed with 30 ml Tris-NaCl buffer at 0.20 ml/min. Bound material was eluted with a 30 ml linear gradient of zero - 80% (v/v) EtOH in Tris-NaCl buffer at 0.20 ml/min, followed by 20 ml of 80% EtOH in Tris-NaCl buffer. Ten µl aliquots of one ml fractions were analyzed for SAA by Dot Blot (Biorad, Richmond, CA) probed with an alkaline phosphatase conjugated monoclonal antibody (Mab) to SAA. SAA positive fractions were combined and concentrated to 1.0 ml via ultrafiltration using a YM-2 (1000 mw cut off) membrane (Amicon, Danvers, MA). This SAA enriched concentrate was then dialyzed in three, 10 volume changes of 10% formic acid in Spectra/Por 3, 3500 mwco dialysis membrane tubing (Spectrum Medical Industries, Los Angeles).

Gel Filtration Chromatography: The SAA concentrate from the Octyl Sepharose CL4B chromatography was injected onto a 1.0 cm X 60 cm column of Sephacryl S-200 (Pharmacia, Piscataway, NJ) which had been preequilibrated with 5 column volumes of 10% formic acid. One ml fractions eluted with 10 % formic acid were collected, neutralized with 2 M NaOH, then analyzed by Dot Blot for SAA. SAA positive fractions were dialyzed overnight in three, 200 ml changes of phosphate buffered saline (PBS) containing 0.01% sodium dodecyl sulfate (SDS).

SDS-PAGE: Materials to be examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoresed on discontinuous gels according to the method of Laemmli (1970) using a 12% acrylamide gel and a 3% acrylamide stacking gel.

SAA Assay: Immulon II (Dynatech Laboratories, Chantilly, VA) microtiter plates were coated with a rat IgG1 Mab to SAA in 100 µl of 48 mM, pH 9.6 carbonate buffer at a concentration of 1.0 µg/well. The plates were incubated at 37° for two hours then washed three times with PBS/.05% Tween-20 (PBS/Tween). Samples diluted 1:2000 in PBS/Tween, or SAA standards, (100 µl) were added to the plate and incubated for 1 hour at 37°. After washing, plates were probed with an alkaline phosphatase conjugated Mab to SAA which binds a separate, non-overlapping epitope of SAA. The plate was washed 3 times with PBS/Tween, and developed using Phosphatase substrate (Sigma, St Louis, MO) in 10% diethanolamine buffer (Harlow and Lane, 1988). Optical densities were determined on a microplate reader at 405 nm (Skatron, Sterling, VA)

N-terminal Amino Acid Analysis: N-terminal amino acid sequence analysis was performed in the Protein Core Facility of the University of Nebraska Medical Center utilizing an Applied Biosystems Model 477A Protein Sequencer with online ABI 120A analyzer.

RESULTS:

Hydrophobic Interaction Chromatography: The elution profile of acute phase serum chromatographed on Octyl Sepharose CL4B is shown in Figure 1. Fractions 54-65 tested positive for SAA by Dot Blot analysis and were combined and concentrated as described in materials and methods. Quantitation of both SAA and total protein showed that this initial step yielded a 59-fold purification with an 89% recovery of SAA (Table 1).

Gel Filtration Chromatography: The elution profile of the SAA enriched concentrate chromatographed on Sephacryl S-200 is shown in Figure 2. Most of the SAA eluted from the Sephacryl S-200 column in fractions 15 and 16 which contained 1720 μg and 360 μg of SAA respectively. These two fractions represented 63% of the SAA applied to the column, for an overall 56% recovery of SAA from serum. SAA comprised 92.3% of the total protein measured in the combined fractions 15 and 16 (Table 1) and was the only protein detectable in a coomassie blue stained SDS-PAGE gel.

N-terminal Amino Acid Sequence Analysis: Five μg of purified SAA was submitted for 15 cycles of amino acid sequence analysis. The sequence determined, R S F F S F L G E A F D G A R, was identical with that determined from SAA cDNA (Sipe et al., 1985; Steinkasserer et al., 1990).

DISCUSSION:

The use of EtOH to elute SAA from Octyl Sepharose CL4B avoided the use of high concentrations of guanidine, and resulted in a smaller sample volume (12 ml as opposed to 60 ml) (Raynes and McAdam, 1988) which was easily and rapidly concentrated for further purification. The use of formic acid as the eluant during the gel filtration step avoided the use of guanidine and yielded most of the SAA in two, one ml fractions easily dialyzed to remove sodium formate. The 56% recovery of SAA improve over that reported by Raynes and McAdam (1988) of 33% overall after phenyl sepharose, gel filtration, and ion exchange chromatography. He reported a higher recovery of SAA from octyl sepharose but did not specify his final recovery for that purification scheme.

The advantages offered by the method reported herein are 1: The toxicity and/or biological activities associated with guanidine and urea (DeDyn and MacDonald, 1990; Windholz et al., 1976) which necessitate exhaustive dialysis before recovered materials can be placed in a biological system is avoided. 2: Purified product is obtained in a high concentration in 2 ml 10% formic acid, a sample volume which is conveniently manipulated. 3: The use of EtOH and formic acid is relatively inexpensive. These methods could be readily employed to permit the purification of other apolipoproteins from human serum.

The amphipathic nature of SAA has been used to effect its rapid, inexpensive purification from serum. The methods reported could be readily scaled up to allow for the purification of larger quantities of SAA from acute phase serum, which will lead to more extensive study of this potentially very important acute phase protein.

ACKNOWLEDGEMENTS:

We thank Dr. Steve Carson and Ms. Jodi Booth for their expert advice and assistance in this undertaking.

Table 1.

Purification of Serum Amyloid A

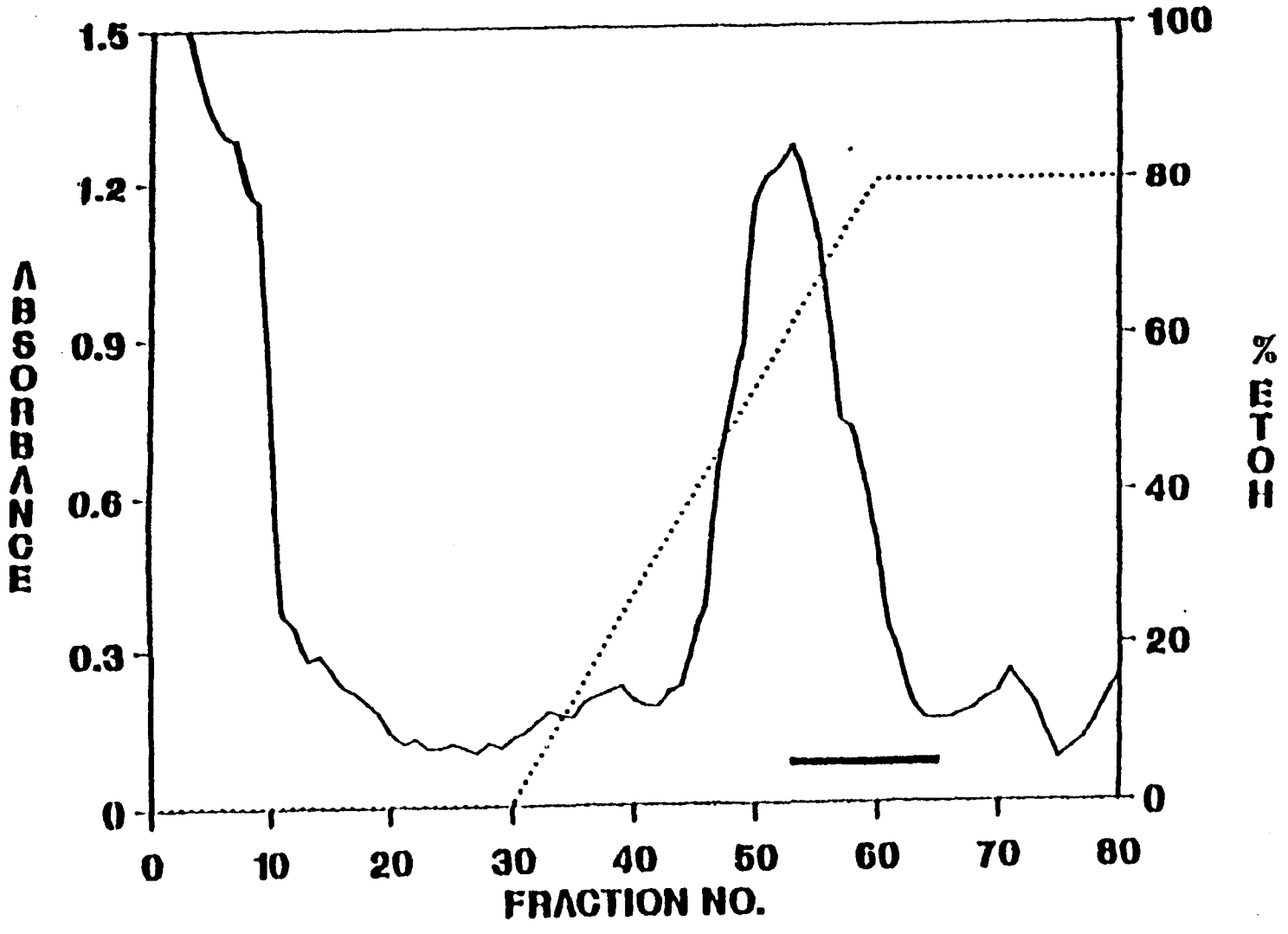
Step	Total Protein (mg)	SAA(mg)	% SAA	Purification (fold)	Recovery(%)
0[a]	1420	3.80	.267	N/A	N/A
1[b]	21.4	3.41	15.93	59	89
2[c]	2.08	1.92	92.3	5.8	63
Overall	2.08	1.92	92.3	342	56

[a]Acute phase serum.

[b]SAA enriched concentrate recovered from Octyl Sepharose CL4b column.

[c]Purified SAA recovered from Sephacryl S-200 column.

Figure 1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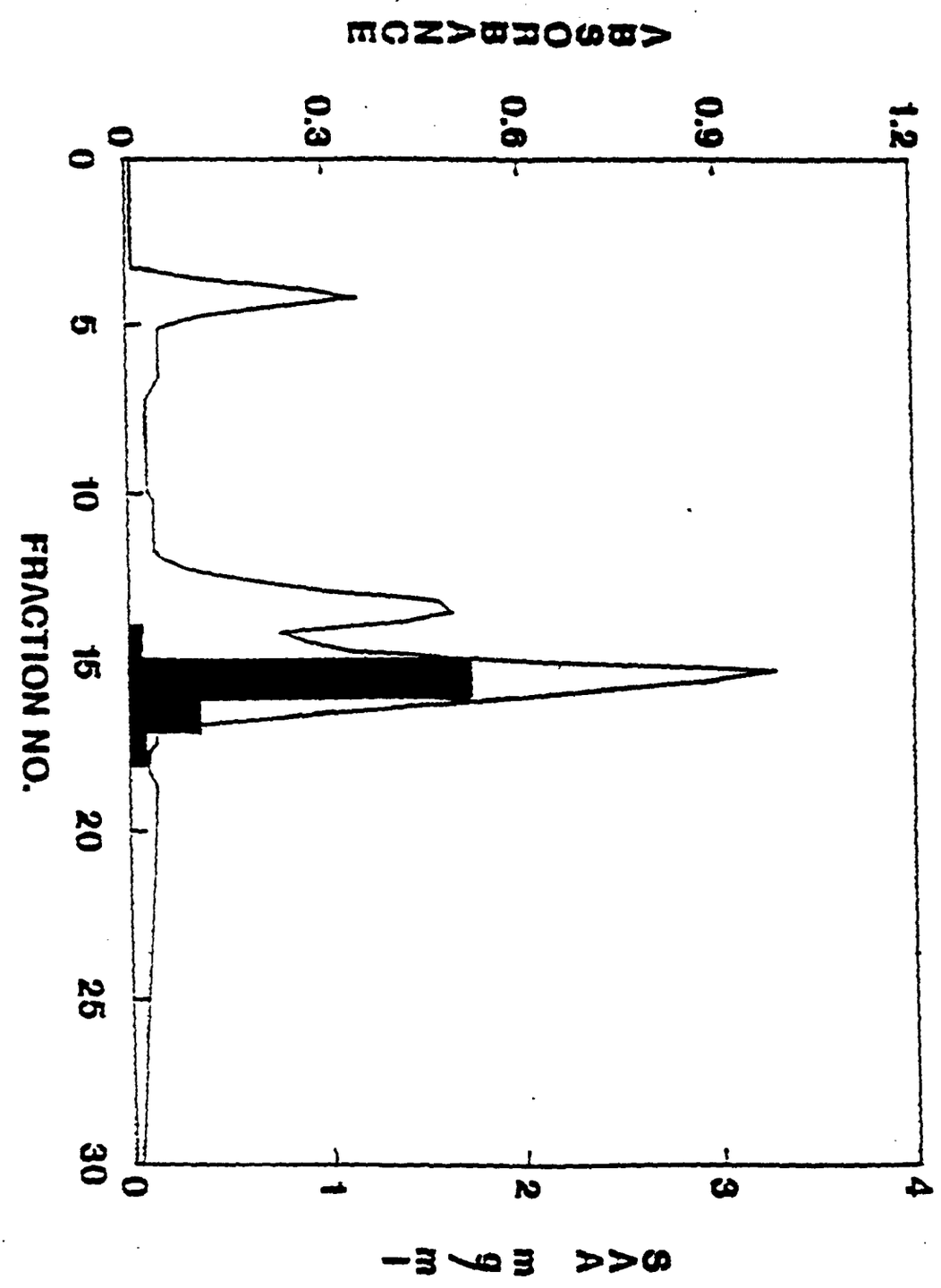
1.5
1.2
0.9
0.6
0.3
0
100
80
60
40
20
0
% ETOH
0
10
20
30
40
50
60
70
80
FRACTION NO.

Figure 2


ABSORBANCE
0
0.3
0.6
0.9
1.2
0
5
10
15
20
25
30
FRACTION NO.
0
1
2
3
4
SAA mg/ml

Legends:

Figure 1: Elution profile of 20 ml acute phase serum containing 3.80 mg SAA chromatographed on a 1.0 X 9.0 cm column of Octyl Sepharose CL4B eluted with a linear gradient of 0 - 80 % EtOH (....). Elution was monitored at 280 nm (——). One ml fractions were collected and analyzed for SAA by Dot Blot (▬▬). Fractions which contained SAA (54-65) were pooled for further purification.

Figure 2. Elution profile of SAA enriched concentrate from Octyl Sepharose column chromatographed on a 1.0 cm X 60 cm column of Sephacryl S-200. Proteins were eluted as described in text with 10% formic acid. Elution was monitored at 280 nm (——). One ml fractions were collected and analyzed for SAA by Dot Blot. SAA positive fractions (14-17) were dialyzed and assayed for SAA by Elisa (■ mg/ml).

Figure 3. SDS-PAGE of materials purified. Lane 1; Aliquot of 20 ml unfractionated serum applied to Octyl Sepharose column. Lane 2: Aliquot of 60 ml non-bound material from Octyl Sepharose column. Lane 3: SAA positive material from Octyl Sepharose column. Lane 4: Fraction 15 from Sephacryl S-200 column. Lane 5: Fraction 16 from Sephacryl S-200 column.

Title: A Monoclonal Antibody Sandwich Immnoassay for Serum Amyloid A (SAA) protein

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Key Words: Serum amyloid A, C-reactive protein, enzyme linked immunosorbant assay, monoclonal antibodies.

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ABSTRACT

An antibody sandwich immunoassay using two purified rat monoclonal antibodies to human serum amyloid A was developed and used to measure serum amyloid A in human serum. The assay was specific, sensitive, reproducible, and reliable and does not require denaturation of the specimen prior to assay. Serum amyloid A purified by hydrophobic interaction chromatography of acute phase human serum afforded a reliable standard for the assay. A significant (r=0.69) correlation for SAA and CRP values was found for 180 samples analyzed.

Abbreviations: SAA: serum amyloid A, CRP: C-reactive protein, SDS: sodium dodecyl sulfate, PAGE: polyacrylamide gel electrophoresis, MAb: monoclonal antibody, Tris-NaCl buffer: 10 mM NaCl, 50 mM Tris, pH 7.6, ELISA: enzyme linked immunosorbant assay, PBS: phosphate-buffered saline, pH 7.2, HDL_3: high density $lipoprotein_3$, FBS: fetal bovine serum

INTRODUCTION

Serum Amyloid A (SAA) is produced by the liver and occurs normally as a trace level constituent in plasma. Plasma concentrations of SAA have been shown to increase as much as 1000-fold, to levels approaching 1 mg/ml for short periods (Kushner, 1982; Sipe et al., 1988) in response to an infectious or non-infectious inflammation. mRNA for SAA has been shown to increase as much as 1000-fold in murine liver within a few hours of an appropriate inflammatory stimulus such as injection of lipopolysaccharide (Morrow et al., 1981; Benditt et al., 1988). This response is presumably mediated by the combined effects of Interleukin-1 and Interleukin-6 (Benditt et al., 1988; Moshage et al., 1988; Sipe et al., 1988; Ganapathi et al., 1988) although the exact nature of this response remains under investigation.

The immunomodulatory effects of SAA in the regulation of the immune response to T-dependent antigens observed by Benson and Aldo-Benson (1979,1982) and Peristeris et al. (1989) coupled with the remarkable increases in concentration of SAA during an inflammatory incident has created an interest in its clinical utility for monitoring inflammation in a variety of patient groups.

SAA has been proposed as a marker for measuring inflammation in renal (Hocke et al., 1989) and liver (Maury and Teppo, 1984) transplant rejection, pulmonary infection in cystic fibrosis patients (Marhaug et al., 1983), rheumatoid

arthritis, secondary reactive amyloidosis, neoplastic diseases (Benson and Cohen, 1979), myocardial infarction (Clifton et al., 1985), and trauma (Choukaife et al., 1989). The need for a rapid, reliable, reproducible, accurate assay for the quantitation of serum amyloid A has become apparent.

A variety of assays for SAA have been developed, most of which require some form of denaturation of the plasma or serum to ensure the complete dissociation of SAA from the HDL_3 fraction of lipoprotein (Marhaug et al., 1983; Sipe et al., 1989). The difficulties associated with the development of assays which require a denaturation step have been noted by several investigators, (Marhaug et al., 1983; Pepys and Baltz, 1983; Benditt et al., 1988; Sipe et al., 1989). Denaturation by heat, acid, or alkali appears to lead to considerable variability in the reproducibility of the assays (Sipe et al., 1989). The recently reported assay of Sipe et al., (1989) requires overnight 60° C incubation in pH 9.6, 3M KBr.

Several antibody sandwich ELISAs have been described, employing rabbit antisera to SAA (Dubois and Malmendier, 1988), to amyloid A purified from an amyloidotic human kidney (Yamada et al., 1989), or to discrete peptides of SAA (Saile et al., 1989), or rat antisera to SAA (Zuckerman and Suprenant, 1986).

Rats do not produce detectable amounts of SAA (Kushner and Mackiewicz, 1987) and are therefore ideal as a source of spleen cells for the production of the high affinity

monoclonal antibodies to separate, non-overlapping epitopes of SAA. The assay described requires no sample denaturation and can be performed in 3 hours.

MATERIALS AND METHODS

Preparation and Purification of Monoclonal Antibodies SAA was isolated from the serum of rheumatoid arthritis patients by electroeluting it from a 12% SDS polyacrylamide gel. Electroelution was done according to manufacturers instructions (Bio-Rad Inc, Richmond, CA). Female Sprague Dawley rats were immunized subcutaneously with 25 μg SAA in an emulsion of 0.5 ml complete Freund's adjuvant. At two weeks and again at four weeks the rats were boosted via subcutaneous injection with an additional 25 μg SAA emulsified with 0.5 ml incomplete Freund's adjuvant. Two weeks after the final injection the rats were sacrificed, spleens removed, and the splenocytes harvested from a percol gradient. Splenocytes were fused at a 1:1 ratio with NS-1 murine myeloma cells in polyethylene glycol using standard methods (Wood et al., 1982). Clones were selected based on production of IgG antibodies with affinity for SAA as detected by Western blotting, visualized with alkaline phosphatase labeled mouse anti-rat IgG Fc(Jackson Laboratories, West Grove, PA).

Selected clones were grown in RPMI 1640 (Gibco, Grand Island, NY) with added glutamine (2.4 mM,) 10% FBS (HyClone, Logan, UT),1% penicillin streptomycin solution (Gibco, Grand

Island, NY), 1% Fungizone solution (Gibco, Grand Island, NY), and 1% Origen solution (Sigma, St Louis, MO), then gradually weaned to 1% FBS, with no Origen added.

The hybridoma fluids were acidified to pH 5.0 with 0.1 M acetic acid, filtered through a 0.45 micron filter (Nalgene, Rochester, NY), then passed through a Protein G column (Genex, Gaithersburg, MD) which had been preequilibrated with pH 5.0, 100 mM acetate buffer, 150 mM NaCl (Genex Technical Assistance). Bound antibody was eluted with 0.50 M pH 3.0 acetate buffer and the eluate was immediately neutralized with 5M NH_4OH.

Alkaline Phosphatase Conjugation of Monoclonal Antibody Purified antibody was dialyzed 48 hours with 3 changes of PBS to remove all ammonium ions. The antibody was conjugated with alkaline phosphatase (Sigma, St. Louis, MO) via a single step procedure utilizing glutaraldehyde (Harlow and Lane et al., 1988). The labeled antibody recovered was used diluted 1:5000 for probing Western blots, and 1:2000 for ELISA.

Determination of Specificity of Monoclonal Antibodies SAA enriched fraction containing approximately 15% SAA (Cal-Biochem, San Diego, CA) was electrophoresed on a 12% SDS-PAGE, and transferred to Immobilon-P (Millipore, Bedford, MA) for Western Blot analysis. The blots were probed with either rabbit polyclonal anti-SAA (CalBiochem, San Diego, CA) or the

purified monoclonal antibodies to determine staining patterns. Acute phase serum from Balb C mice which had been used for ascites production or from humans with a variety of inflammatory conditions was electrophoresed and Western blots were probed with each of the anti-SAA MAbs produced by the previously selected clones.

Preparation of SAA Standard Seventy ml of human serum containing SAA, as determined by Western blot analysis, was pooled and adsorbed onto a 2.0 cm X 30 cm column of Phenyl Sepharose CL-4B (Pharmacia, Piscataway, NJ) (Raynes and McAdam, 1988) previously equilibrated with Tris-NaCl buffer. The column was washed with 600 ml Tris-NaCl buffer to remove nonbound material, then eluted using a zero to 80% (w/v) linear gradient of propylene glycol (Sigma, St. Louis, MO) in Tris-NaCl buffer (600 ml total gradient volume) followed by 300 ml 80% propylene glycol in Tris-NaCl buffer. (Ross and Carson, 1985). Five ml fractions were collected. A 25 μl aliquot of each fraction was assayed for SAA by dot blot (BioRad, Richmond, CA) probed with the alkaline phosphatase labeled MAb. SAA positive fractions were combined, dialyzed for 24 hours with 3 changes of 40 mM PBS, then concentrated by ultrafiltration using a YM2 (1000 mw cutoff) filter (Amicon, Danvers, MA). The concentrated material was electrophoresed on a 12% SDS PAGE, silver stained, and SAA percentage (12 kDa band) was determined by

scanning transmittance densitometry (Hoeffer Scientific Instruments, San Francisco, CA) as previously reported by Sipe et al. (1989). SAA concentration (% SAA X total protein concentration as determined spectrophotometrically (Groves et al., 1968) was adjusted to 500 μg/ml with addition of PBS. This solution was used as the standard for quantitating SAA in test specimens.

Assay Procedure The non-conjugated MAb 6B10 was coated on microtiter plates (Dynatech Laboratories, Chantilly, VA) at a concentration of 1 μg/well for two hours at 37° C in 48mM carbonate buffer, pH 9.6. Plates were washed three times with PBS/.05% Tween 20, then stored up to 3 weeks in PBS/.05% Tween 20/ 0.01% sodium azide at 4° C. Serum was warmed to room temperature for a minimum of two hours, thoroughly vortexed, then diluted 1:250 in PBS. Ten μl diluted serum or standard was added to triplicate microtiter plate wells containing 90 μl PBS/.05% Tween 20, to make the final dilution 1:2500. The plate was incubated for one hour at 37° C, then washed three times with PBS/.05% Tween 20. The conjugated MAb 5G6 was added (100 μl of a 1:2000 dilution in PBS/.05% Tween 20) and the plate incubated one hour at 37° C. The plate was washed three times with PBS/.05% Tween 20, and Sigma 104 phosphatase substrate tablets (p-nitrophenyl phosphate) (Sigma, St. Louis, MO) dissolved in 1.0 M diethanolamine, pH 9.8 with 0.1 g/l $MgCl_2.6H_2O$, to yield a solution of 1.0 mg/ml p-nitrophenyl

phosphate, was added. After one hour incubation the optical density at 405 nm was measured on a microplate reader (Skatron, Inc.,Sterling,VA). Standards were included on each plate. SAA concentration of test specimens were calculated from a standard curve generated with known concentrations of SAA.

Patients The study population consisted of 180 patients from whom blood had been collected for CRP determinations. No additional selection criteria were used. An aliquot of serum from each patient was obtained and stored at -20° C until assayed for SAA.

RESULTS

Epitope Specificity of Monoclonal Antibodies In order to design an assay such as the above described, it was necessary to select monoclonal antibodies to distinct, nonoverlapping epitopes of SAA. Figure 1. shows that the MAb 5G6 binds both human and murine SAA, while the MAb 6B10 binds only the human SAA. This indicates that 5G6 binds a region of SAA among that portion which is highly conserved between murine and human SAA (Benditt et al., 1988), while the 6B10 binds a region not so highly conserved.

Assay Conditions The calibration curve for the SAA assay is shown in figure 2. The coefficients of variation for the standard curve ranged from 3.4% to 10.3%, average 6.5% (Table 1). When plates were coated with a MAb unrelated to SAA instead of the mAb 6B10, then the serum was assayed, no binding was detected. Ten serum samples with SAA concentrations ranging from 5 to 420 μg/ml were assayed by the reported method after prior denaturation at 60° C for 2 hrs in sealed microcentrifuge tubes. Concentrations determined were 102 % (range 89 - 113 %) of the values determined by the reported assay performed on non-denatured samples. The same ten samples were assayed by the method of Taktak et al., (1991), which employs overnight coating at 4° C of serum samples diluted in pH 9.6 carbonate buffer, and by the method of Sipe et al., (1989) which uses overnight coating at 60° in 3.0 M KBr, pH 9.6 carbonate buffer. Concentrations determined were 103 % (range 91-122 %), and 92 % (range 63-108%), respectively, of the values determined by the reported assay performed on non-denatured samples. Two samples had SAA values of <15 μg/ml and were not quantifiable by the methods of Taktak or Sipe in this investigators hands, thus were not included in calculations, whereas all 10 samples were measurable in the MAb sandwich assay.

Reproducibility and Recovery The intra-assay coefficients of variation obtained from repeat analyses of 4 serum samples with SAA concentration of 74-1030 μg/ml ranged from 2.7% to 7.7%, average 4.9%. The inter-assay coefficients of variation for these same samples ranged from 3.8% to 10.8%, average 7.8% (Table 2). The range of the assay is from 5 to 500 μg/ml. Test samples which had a concentration greater than 500 μg/ml were diluted 1:500 in PBS (instead of 1:250) and re-assayed. Serum samples were stable through multiple freeze-thaw cycles, at 4° C for 30 days, and for 6 months at -20° C (Data not shown.) SAA stock solution was added to sera which had tested negative for SAA by ELISA and by SDS-PAGE and Western Blot. Samples were vortexed, allowed to stand at room temperature for two hours, then vortexed again and assayed. SAA was fully accounted for (average recovery of 98.3%) when added to several SAA negative sera (Table 3).

Correlation of SAA and C-Reactive Protein Concentrations C-Reactive Protein measurements were determined in the clinical laboratory at the University of Nebraska Medical Center by laser immunonephelometry using a Behring Nephelometer-Analyzer (Behring Diagnostics, Somerville, NJ). The normal range for CRP in the clinical laboratory is <6 μg/ml. The normal range for SAA was arbitrarily set at <15 μg/ml. The correlation between CRP and SAA is shown in figure 4. The calculated Pearson's correlation coefficient was 0.69.

DISCUSSION

The effect of anti-inflammatory therapy is most effectively monitored by the measurement of acute phase proteins. C-reactive protein is the most utilized assay for this purpose, but SAA, due to its greater incremental range, is a more sensitive indicator of inflammation (Janssen et al., 1986). It has been suggested that the regulation of SAA and CRP serum concentrations may be by independent, disease-specific pathways, (Schultz and Arnold, 1990). However, it appears that in most diseases SAA is a more sensitive indicator of inflammatory stimuli than CRP, but does not demonstrate disease specificity (Schultz and Arnold, 1990; Husbekk et al., 1986; Maury et al., 1984; Marhaug et al., 1983). Because SAA is such a sensitive indicator of both infectious and non-infectious inflammation, it is of clinical interest to have available a rapid, accurate assay for its measurement in serum.

The advantages of the two MAb sandwich immunoassay as described are: (1) No denaturation of the specimen is required. Denaturation of samples by heating at 60° for 2 hours prior to analysis had no effect on the assay. (2) The hybridomas producing the monoclonal antibodies are self-renewing, relatively inexpensive to maintain, and yield a highly consistent, easily purified product. (3) The microtiter plates can be coated and stored, making the assay available within a relatively short time (3 hours). (4) The assay is

accurate and reproducible. In this investigators hands, the reported assay proved more sensitive for detection of minimally increased concentrations of SAA than two other assays to which comparisons were made. (5) The assay requires only equipment and skills which are normally available in either a research or a clinical laboratory.

The SAA solution prepared via hydrophobic interaction chromatography on Phenyl Sepharose CL-4B afforded a reliable, stable standard for use in the assay. SAA was purified to homogeneity by gel filtration in 10% formic acid, and N-terminal amino acid sequence data consistent with previously published data was obtained (Smith and McDonald, 1991). This product was found to be unsuitable for standardization of the immunoassay, presumably because of conformational changes incurred during the purification procedures, and resultant loss of required conformational epitopes of SAA. Similar findings were reported by Dubois and Malmendier (1988).

In summary, the double antibody sandwich immunoassay using two monoclonal antibodies offers a reliable, reproducible, accurate assay for the measurement of SAA in those patients where such measurement might prove clinically valuable. The assay could easily be adapted for use in the clinical laboratory.

Table I.

Reproducibility of Standard Curve

Plate #	SAA Concentration					
	500	300	200	100	50	25
	Optical Density at 405 nm					
1	.826	.580	.482	.300	.172	.125
2	.815	.590	.463	.280	.155	.110
3	.904	.632	.502	.312	.180	.130
4	.815	.577	.445	.247	.161	.107
5	.764	.577	.422	.244	.148	.101
6	.826	.584	.461	.276	.180	.125
Mean	.826	.592	.463	.277	.161	.116
SD	.041	.020	.028	.027	.012	.012
CV(%)	4.9	3.4	6.0	9.7	7.4	10.3

Table II.

Intra and Inter-experimental variation analysis of SAA Assay

Intra-assay (n=15)			Inter-Assay (6 independent determinations)		
Mean (μg/ml)	SD	CV(%)	Mean (μg/ml)	SD	CV(%)
1030	28.0	2.7	1048	40.0	3.8
312	12.3	3.9	308	25.1	8.1
128	6.8	5.3	134	11.7	8.7
74	5.7	7.7	81	8.7	10.8

Table III.

Recovery of SAA.

Added SAA (μg/ml)	Recovered SAA (μg/ml)	Recovery (%)
300	320 ± 23.0	107 ± 7.2
250	270 ± 14.6	108 ± 5.4
150	140 ± 7.0	93 ± 5.0
125	120 ± 6.9	96 ± 5.8
75	70 ± 2.9	93 ± 4.2
37	40 ± 1.7	108 ± 7.3
18	15 ± 1.8	83 ± 12.0

Figure 1

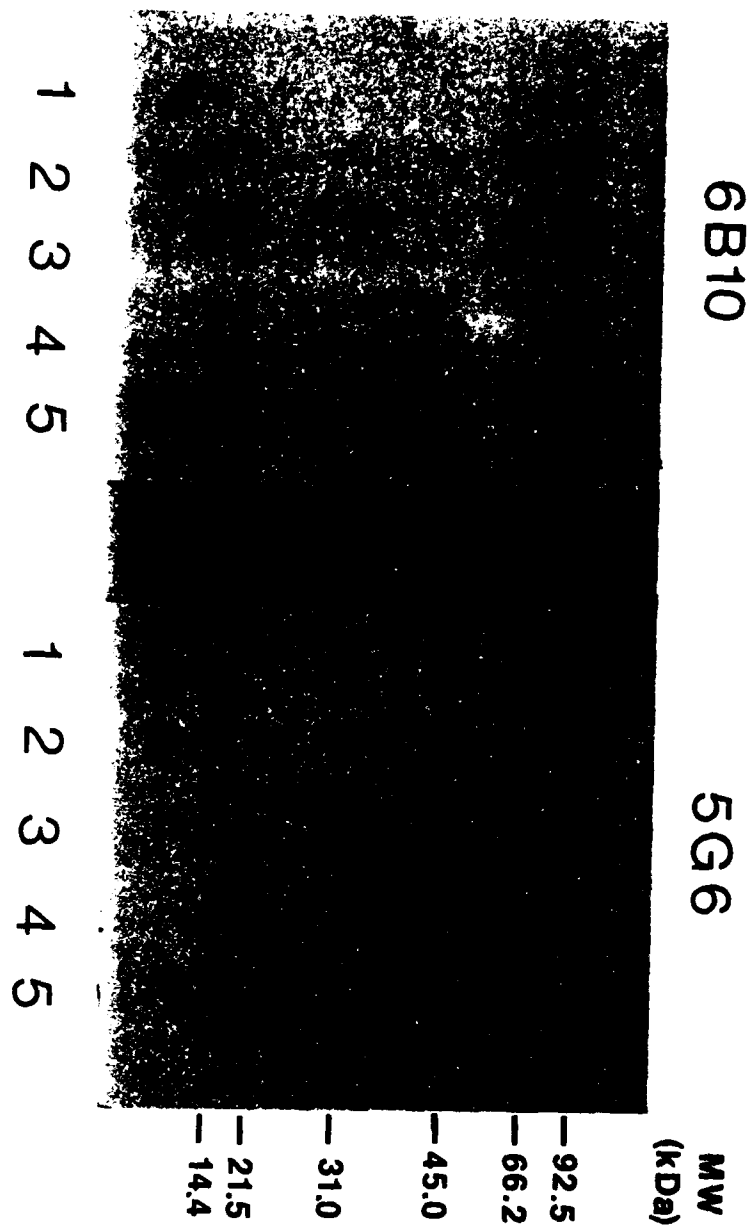


Figure 2

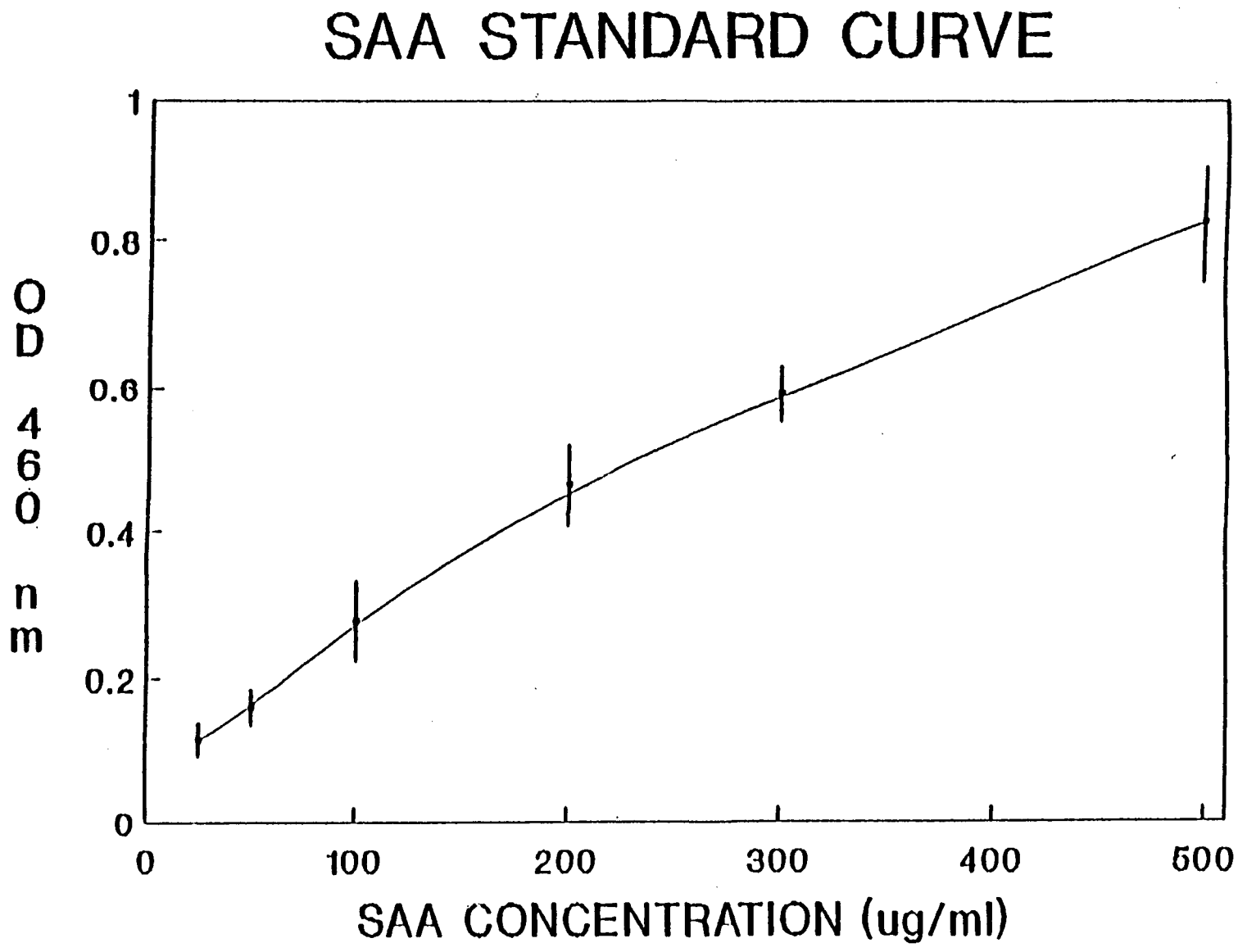
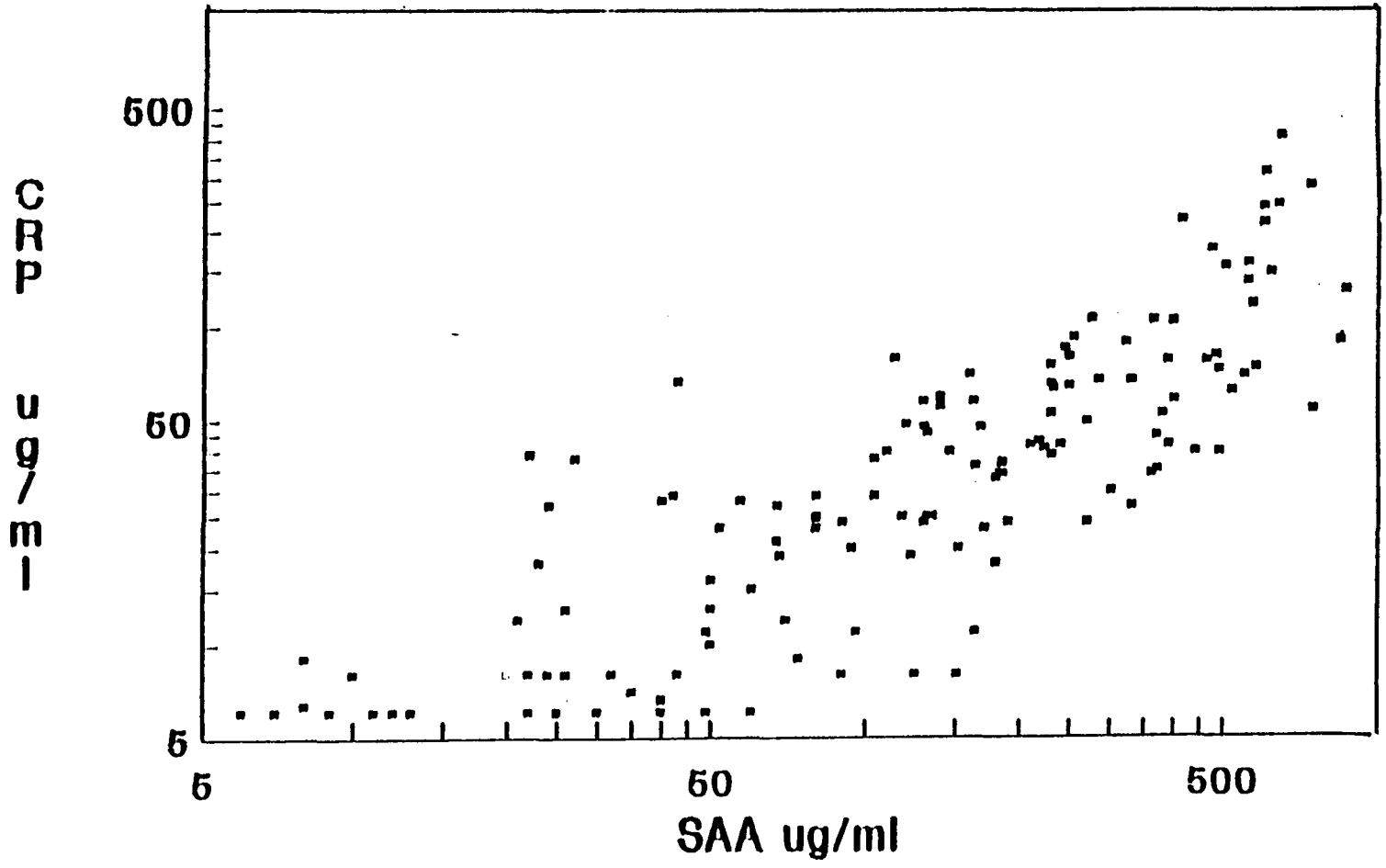

SAA STANDARD CURVE
OD 460 nm
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
SAA CONCENTRATION (ug/ml)

Figure 3

CORRELATION OF SAA WITH C-REACTIVE PROTEIN



Legends (Tables)

Table 1. Standard curve reproducibility. Standard curves were determined on six different days to assess reproducibility. Assay was as described in materials and methods section. All points are reported as the mean of triplicate determinations.

Table 2. Assay precision and recovery experiments. Four serum samples were assayed 15 times on a single plate as described in materials and methods section (intra-assay) or in triplicate on six different days (inter-assay).

Table 3. Recovery experiments. SAA was added to an SAA negative serum, vortexed, allowed to stand for two hours at room temperature, then assayed as described in materials and methods section.

Legends (Figures)

Figure 1. Western blot of SDS-PAGE of human and murine serum probed with mAb 6B10 and 5G6. 12% gels were loaded with 1 μl serum diluted in PBS and non-reducing loading buffer. After electrophoresis protein was transferred to Immobilon-P on a BioRad semidry blotter and probed with either mAb 6B10 or mAb 5G6. Lane 1: SAA standard (0.5 μg SAA). Lane 2: Acute phase serum, Patient A (SAA 310 μg/ml). Lane 3: Acute phase serum, Patient B (SAA 130 μg/ml). Lane 4: Normal human serum (SAA <5 μg/ml). Lane 5: Murine acute phase serum.

Figure 2. SAA standard curve. SAA standard (500 ug/ml SAA) was diluted in PBS to various noted concentrations, and assayed in triplicate. Mean ± 2 SD for triplicate determinations of each concentration assayed is indicated. Average coefficient of variation was 6.5%.

Figure 3. Correlation of C-reactive protein with SAA. CRP and SAA were assayed as described in serum samples from 180 patients.

Title: Comparison of Serum Amyloid A and C-reactive Protein as Indicators of Lung Inflammation in Corticosteroid Treated and Non-corticosteroid Treated Cystic Fibrosis Patients

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Running Title: SAA and CRP Levels in Cystic Fibrosis Patients

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Key Words: Acute phase reactants, acute phase response, glucocorticoids, prednisone, prednisolone, pulmonary function

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ABSTRACT

Serum amyloid A (SAA) and C-reactive protein (CRP) levels were compared in 830 serum samples from 155 cystic fibrosis (CF) patients. Correlation coefficients were calculated for all samples ($r=0.73$), for samples from non-corticosteroid treated (CFNS) patients ($n=698$, $r=0.80$) and for samples from corticosteroid treated (CFS) patients ($n=132$, $r=0.35$). SAA was the more sensitive indicator of pulmonary inflammation when SAA and CRP were compared to pulmonary function tests of 49 hospitalized patients at admission and discharge. CRP levels were significantly ($p<.05$) lower at admission in CFS patients than in CFNS patients, whereas SAA levels were not significantly different between the two groups. All nine CFS patients hospitalized had elevated SAA levels (average 22 times above normal limits) at admission, while only six had elevated CRP levels (average 3.7 times above normal limits) at admission. In the 40 CFNS patients both SAA and CRP levels were significantly elevated at admission. In each case SAA and CRP levels declined as pulmonary functions improved with effective antimicrobial therapy. In three instances SAA levels increased during hospitalization while CRP levels did not. In each case, rising SAA levels indicated clinical deterioration associated with evolving resistance of *P. aeruginosa* which required a change in antibiotic therapy.

INTRODUCTION

Pulmonary dysfunction, primarily due to chronic airway infections, is responsible for approximately 90% of the mortality in cystic fibrosis (CF) patients (Gilligan, 1991; Thomassen et al., 1987a). Commonly CF patients exhibit a cycle of relative well-being that is repeatedly complicated by episodes of recurrent pulmonary infections. These recurrent infections are characterized by symptoms such as fever, weight loss, anorexia, increased cough, increased respiratory rate, and the appearance of new lung infiltrates detected by chest radiographs. With each exacerbation pulmonary function progressively declines, eventually leading to pulmonary failure and death (Gilligan, 1991).

Pseudomonas aeruginosa is clearly the most important pathogen to be considered in the treatment of pulmonary exacerbations associated with cystic fibrosis (Gilligan, 1991). P. aeruginosa is found in the sputum of 70% to 90% of CF patients (Fick, 1989; Hoiby, 1988). The isolate is usually a mucoid form of P.aeruginosa which is able to evade the normal host defense mechanisms of the lung and grow to concentrations of greater than 10^8 bacteria per ml sputum (Gilligan, 1991; Hoiby, 1988; Thomassen et al., 1987b; Levy, 1988). Despite the use of potent anti-pseudomonal antibiotics, the organism is rarely eradicated from the respiratory tract of CF patients, and is often the only pathogen isolated from

sputum or lung tissue obtained by thoracotomy or at autopsy (Fick, 1989).

The genetic defect responsible for most cases of cystic fibrosis has recently been characterized (Rommens et al., 1989; Kerem et al., 1989), but the mechanism leading to the chronic obstruction of the airways and the initiation of a life threatening cascade of inflammatory processes remains unclear. The destruction of lung structure and function is associated with the release of inflammatory substances such as elastase, activation of complement components, and production of immunoglobulins (Doring et al., 1988; Saak et al., 1990). Immunoglobulins combine with P. aeruginosa in the lung and form immune complexes which are not phagocytosed or otherwise cleared from the lung. These immune complexes contribute to the sequelae associated with P. aeruginosa infections (Gilligan, 1991; Fick, 1989; Doring et al, 1988). The use of steroidal anti-inflammatory therapy to control the excessive inflammatory response in these patients has gained some support in recent years (Kerrebijn, 1989; Auerbach, 1989). Auerbach (1989) showed in a four year study that CF patients who received alternate day prednisolone therapy required fewer hospitalizations and had significant advantages over the placebo group in terms of height, weight, pulmonary function, erythrocyte sedimentation rate, and serum IgG levels. A long-term prospective study on the use and effects of corticosteroids in CF patients is currently underway in the

United States and Canada, but results are not yet available (Kerrebijn, 1988).

Effective medical management directed at symptoms of CF airway disease, including aggressive antimicrobial therapy for recurrent infections combined with early recognition and diagnosis of the disease, have increased the mean survival age of CF patients to 26 years (Fick, 1989). However, discerning P. aeruginosa colonization from active infection may be difficult or impossible on the basis of quantitative sputum cultures (Levy, 1988; Nelson, 1985; Glass et al., 1988). Patient assessments based on chest radiographs, weight loss, anorexia, erythrocyte sedimentation rate, leukocyte count and pulmonary function tests, while useful, may provide variable results which do not always accurately determine patient status. In patients with significant loss of lung function, or in very young (under 7 years) patients who may be less than fully cooperative in performing pulmonary function tests, there are few objective measurements on which to base a decision to initiate antimicrobial therapy (Glass et al., 1988; Rayner et al., 1991).

C-reactive protein (CRP), an acute phase reactant produced by the liver in response to interleukin (IL)-1 and IL-6, (Kushner et al., 1989) is useful as a rapid and reliable measure of inflammation in a variety of diseases including pulmonary inflammation associated with cystic fibrosis (Saak et al., 1990; Glass et al., 1988; Rayner et al., 1991; Moreton

and Kennedy, 1988; Heininger et al., 1991). Glass et al. (1988) showed that CRP levels above 10 mg/liter were associated with pulmonary exacerbation in CF patients, and the return of CRP to normal levels coincided with clinical resolution of the exacerbation. Elevated CRP levels correlated with fever, weight loss, leukocytosis, and a decline in pulmonary function (Saak et al., 1990; Rayner et al., 1991), and returned to normal or near-normal levels after effective antimicrobial therapy (Saak et al., 1990; Glass et al., 1988; Rayner et al., 1991; Heininger et al., 1991). These observations have led to the increased use of CRP measurements as an index of acute or impending pulmonary exacerbation in CF patients, and as a means to monitor the efficacy of antibiotic therapy.

Serum amyloid A (SAA) is a low molecular weight (12kDa) protein which is produced primarily by the liver in response to IL-1, IL-6, and tumor necrosis factor (TNF) (Sipe et al., 1988). SAA can reach a concentration of up to 1.0 mg/ml (100 - 1000 times the normal physiological level) in the sera of acutely ill patients (Schultz and Arnold, 1990). SAA has been demonstrated to have utility in predicting renal allograft rejections (Maury and Teppo, 1984) and in assessing the inflammatory status of rheumatoid arthritis (Benson and Cohen, 1979) and trauma patients (Mozes et al., 1989) . SAA also was shown to be more sensitive than CRP or α-1 acid glycoprotein as an indicator of lung inflammation in CF patients (Marhaug

et al., 1983) and increased levels of SAA correlated with the presence of pathogens in the sputum. Marhaug et al. (1983) showed that a decrease in the concentration of SAA during antimicrobial therapy was a good indicator of efficacious therapy, though frequently the concentration of SAA never returned to normal levels in these patients. Efforts to ascertain the clinical utility of SAA as an effective measure of inflammation have been hampered by the lack of a commercially available assay for SAA which could be utilized for the large numbers of samples required for such a study (Chambers et al., 1991). The aims of this study were three-fold: First, to determine SAA concentrations in the sera of CF patients using a newly developed immunoassay (McDonald et al., 1991; Smith and McDonald, 1991) and to compare those levels to CRP levels in the same samples; second, to correlate both CRP and SAA levels with pulmonary function as determined by Peak Expiratory Flow Rate (PEFR), Forced Vital Capacity (FVC) and Forced Expiratory Volume in 1 second (FEV1); and finally, to assess the effect of corticosteroid anti-inflammatory therapy on levels of CRP and SAA in this patient population.

MATERIALS AND METHODS

Patients: Excess serum was acquired from blood that was routinely drawn from CF patients for CRP or drug level determinations. A total of 830 samples drawn from 155 patients were

obtained. Samples collected were stored at -20° until assayed for SAA. Corticosteroid treated CF (CFS) patients (5 male, 4 female; median age 23, range 12 - 37 years) received prednisone or methylprednisolone, 1-2 mg/kg, given as a single oral dose on alternate early mornings. CF patients not treated with corticosteroids (CFNS) (19 male, 21 female; median age 16, range 4 - 43 years) did not receive systemic corticosteroid therapy at any time during this study. This study was approved by the Institutional Review Board of the University of Nebraska Medical Center.

SAA Assay: The sandwich immunoassay used has been recently described (McDonald et al., 1991). Briefly, two monoclonal antibodies to nonoverlapping epitopes of SAA were used, one immobilized on ELISA plates to capture SAA from diluted serum, and the other, labeled and used as a detector for captured SAA. Quantitation was interpreted from a standard curve constructed with known concentrations of SAA (Smith and McDonald, 1991). The intra-assay coefficients of variation obtained from repeat analyses of serum samples ranged from 2.7% to 7.7%. The inter-assay coefficients of variation ranged from 3.8% to 10.8%. The range of the assay is from 5 to 500 µg/ml. Test samples which had a concentration greater than 500µg/ml were diluted and reassayed. The antibodies, purified SAA, standards, and the SAA assay are available from BioSource International, Camarillo, CA.

CRP Assay: CRP measurements were determined in the clinical laboratory at the University of Nebraska Medical Center by laser immunonephelometry using a Behring Nephelometer-Analyzer (Behring Diagnostics, Somerville, NJ).

Pulmonary Function Tests: FVC, FEV1, and PEFR were measured with a pneumotach spirometry apparatus (Cybermedic, Inc., Boulder, CO)

Statistics: Statistical comparisons were made using the Student's t-test (two-tailed). Correlations were performed by standard least squares analysis. Analysis was done on a personal computer using the Epistat Statistical Package, version 2.0.

RESULTS

SAA and CRP showed a strong ($r=0.73$, $p<.01$) correlation when all 830 CF patient samples were analyzed as one group (Figure 1). It was observed that there was a clustering of 43 data points in which SAA levels were elevated above normal levels while CRP levels were within normal limits. Thirty five of these 43 samples were from CFS patients. Accordingly, the data was reanalyzed with the CFS and CFNS samples treated as independent groups. When samples from only CFNS patients were included in the data analysis, the Pearson's correlation coefficient indicated an even stronger association ($r=0.80$,

p<.01, n=698) between CRP and SAA. In contrast, the association for samples from CFS patients analyzed as an independent group was much weaker (r=0.35, p=.04, n=132). These disparate correlations suggest that corticosteroid therapy may significantly influence CRP levels in this patient population.

Forty-nine CF patients (40 CFNS, 9 CFS) were hospitalized during this study for seven or more days for intensive antibiotic treatment of pseudomonal lung infections. The patients' SAA and CRP levels were determined at admission and again at discharge and correlated with pulmonary function tests. All nine CFS patients had elevated SAA levels at admission while only 6 had elevated CRP levels. SAA decreased from an average admission level of 226 μg/ml (22 time above normal level limits) to 36 μg/ml at discharge as FVC, FEV1, and PEFR increased significantly. CRP, by contrast, exhibited a much less dramatic decline from 18.6 μg/ml (3.7 times normal limits) at admission to 4.8 μg/ml at discharge (Table I).

In CFNS patients there was a close association between SAA and CRP when evaluated at admission in that all had elevated SAA levels and all but one had elevated CRP levels. Both SAA (average admission value of 324 μg/ml) and CRP levels (average admission value of 55.0 μg/ml) decreased concomitantly during hospitalization as pulmonary functions improved (table I).

There was no statistical difference in pulmonary function tests between the two patient groups at either admission or at

discharge. Regardless of whether or not the patients were receiving systemic corticosteroid therapy, the two patient groups demonstrated equivalent pulmonary function when compared at either admission or at discharge, as well as equivalent improvement in pulmonary function when treated for pseudomonal lung infections. In fact, the only significant difference found in these measurements was that CRP was significantly ($p<.05$) lower in CFS patients at admission than in CFNS patients at admission.

Serial samples from hospitalized patients were analyzed to determine whether SAA and CRP level reflected progressive patient improvement as indicated by pulmonary function tests. Figure 2a illustrates the data from a representative CFS patient who had significant improvement in clinical status during hospitalization. The SAA level was indicative of pulmonary inflammation which resolved with intensive antibiotic therapy while CRP was not. Figure 2b illustrates the data from a similar hospitalization of a CFNS patient in which both SAA and CRP level were indicative of pulmonary inflammation. In three instances (one of which is illustrated in figure 2c) SAA levels increased during hospitalization while CRP levels remained the same or decreased. In each case, evolving resistance patterns of <u>P. aeruginosa</u> was associated with clinical deterioration and required a change in antibiotic therapy to control the infection.

DISCUSSION

Improved effectiveness of antimicrobial therapy for pulmonary exacerbations has contributed to the lengthened lifespan of CF patients. Monitoring the acute phase response of these patients as a means to assess pulmonary inflammation can aid in determining when to initiate antimicrobial therapy as well as providing an accurate indication of the efficacy of therapy employed. C-reactive protein has been utilized as a measure of pulmonary inflammation in CF patients (Saak et al., 1990; Glass et al., 1988; Rayner et al., 1991; Moreton and Kennedy, 1988; Heininger et al., 1991), and is currently used in that context in several regional cystic fibrosis clinics (personal communications). The use of CRP measurements has proven useful for assessing the pulmonary inflammation in most CF patients. Our data is in agreement with that of Marhaug who demonstrated that SAA appears to be more sensitive than CRP as a measure of pulmonary inflammation. In addition, our data indicates that CRP levels may be unsuitable as a measure of pulmonary inflammation in CF patients receiving corticosteroid therapy. CRP levels accurately indicated pulmonary inflammation only in those CF patients who were not receiving corticosteroid therapy whereas serum concentrations of SAA consistently increased or decreased coincident with lung inflammation regardless of treatment. This is supported by the finding that 35 of 43 samples with elevated SAA and normal CRP levels were from individuals who were being treated

with systemic corticosteroids. This finding could explain a similar observation in the study reported by Marhaug et al. (1983) wherein 25% of the CF patients infected with P. aeruginosa had elevated SAA levels but normal CRP levels. Unfortunately, the extent of utilization of systemic corticosteroid anti-inflammatory therapy in that study was not reported.

The nature of the differences between levels of CRP and SAA is unknown at this time. Recent studies have reported elevated levels of IL-1α and IL-1ß in both the plasma and bronchoalveolar washings of infected CF patients (Sutter et al., 1989; Wilmott et al., 1990). Corticosteroids have been demonstrated to reduce the production of IL-1 and IL-6 by monocytes (Bertini et al., 1989; Akira et al., 1990). A possible explanation for the differences observed would be that while both SAA and CRP are synthesized by the liver in response to IL-1, IL-6 and TNF the shorter induction time and higher synthesis rate of SAA (Schultz and Arnold, 1990) may make its production far more apparent in the serum of individuals with low plasma levels of IL-1 and IL-6.

Another potential explanation is that the presence of corticosteroids may induce hepatocytes to increase the production of SAA disproportionately to the level of CRP production. It has been observed that the presence of corticosteroids in tissue culture media induces cytokine stimulated hepatoma cells to disproportionately increase the production of SAA compared to CRP (Kushner et al., 1989). In

freshly isolated human hepatocytes stimulated with IL-6, SAA production was increased by dexamethasone while the production of CRP was unaffected (Castell et al., 1988). These observations lend credence to the hypothesis of a disproportionate production of SAA in the presence of high concentrations of corticosteroids. The mechanism of the observed disparate levels of SAA and CRP in CF patients, however, remains speculative at this time.

Monitoring serum concentration of SAA was shown to be a useful means of evaluating the efficacy of antibiotic therapy (Marhaug et al., 1983). In our study population of hospitalized CF patients, the SAA levels of three individuals increased while CRP levels did not. In each case developing resistance patterns of the P. aeruginosa forced an adjustment in the antibiotic therapy employed in order to effectively control the microorganism.

In summary, the greater incremental range of SAA, as measured by our assay, makes SAA more sensitive than CRP as a measure of pulmonary inflammation in CF patients. Also, it appears that the concentration of SAA is an accurate measure of pulmonary inflammation, and is clinically useful as part of the overall clinical assessment of CF patients. Furthermore, determination of SAA concentrations appear to have a valuable role in the assessment of pulmonary inflammation in CFS patients, where CRP concentrations may be unsuitable for such assessment. In other disease states in which systemic

corticosteroid therapy is extensively utilized, it may be that SAA levels are equally valuable in assessing the inflammatory status of the patient.

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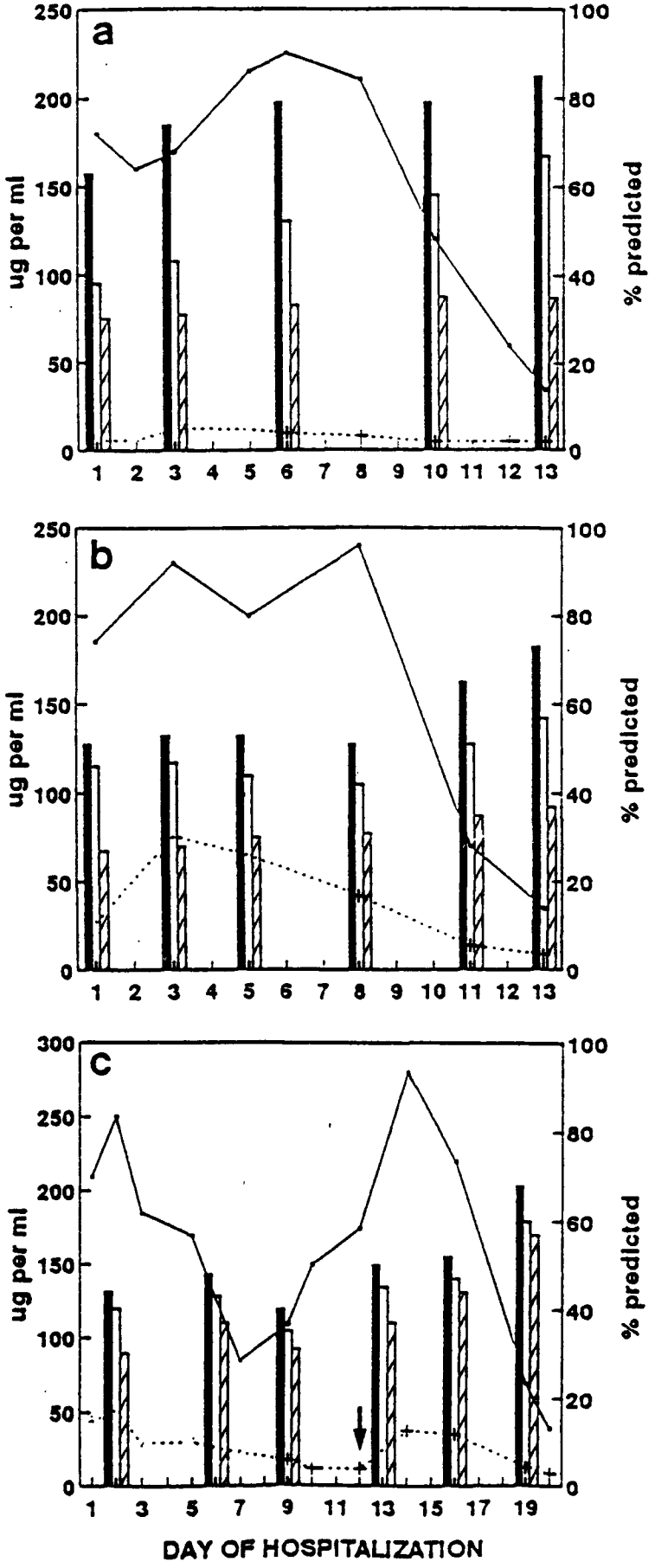
Table I. Comparison of SAA and CRP levels and pulmonary function (FVC, FEV1 and PEFR) of CFS and CFNS patients at admission and discharge.

	CFS Patients (n=9)		CFNS Patients (n=40)	
	Admit	Discharge	Admit	Discharge
FVC	61.2 (8.0)	79.1 (6.5)	54.1 (14.1)	77.1 (19.2)
FEV1	37.4 (12.1)	44.8 (11.3)	33.8 (16.2)	51.8 (20.1)
PEFR	49.3 (11.6)	62.6 (12.0)	58.4 (12.7)	76.4 (20.3)
CRP	18.3 (12.6)	4.8 (1.9)	55.0 (17.8)	7.1 (2.4)
SAA	226 (158)	36.0 (18.4)	324 (212)	33.1 (17.0)

Values are expressed as mean (SD) for % predicted values for FVC, FEV1, and PEFR, and as μg/ml for CRP and SAA. Only patients hospitalized a minimum of 7 days for treatment of lung infections caused by P. aeruginosa are included.

Figure 1

Figure 2



LEGENDS

Figure 1. Comparison of SAA and CRP levels in CF patients. Values are plotted as μg/ml CRP (vertical axis) vs μg/ml SAA (horizontal axis). Solid lines define normal limits, 5μg/ml for CRP, 10 μg/ml for SAA. Dotted lines describe limits defined by correlation parameters ± 2 SD. Individual points may represent more than one sample.

Figure 2. Comparison of SAA and CRP levels with FVC, FEV1, and PEFR. SAA (solid line) and CRP (dotted line) levels are compared to FVC (shaded bars), PEFR (open bars) and FEV1 (hatched bars). a. A representative (1 of 9) CFS patient hospitalized for intensive antibiotic therapy of a lung infection caused by pseudomonas aeruginosa. b. A representative (1 of 40) CFNS patient hospitalized under similar circumstances. c. A patient in whom clinical deterioration associated with evolving resistance of the pseudomonas causing the lung infection necessitated a change (↓) on day 12 in the antibiotic regimen employed.

Title: Production of Serum Amyloid A and C-reactive Protein by HepG2 Cells Stimulated with Combinations of Cytokines or Monocyte Conditioned Media: The Effects of Prednisolone.

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SUMMARY

The hepatic production of the acute phase proteins in response to inflammatory cytokines, and the interaction of corticosteroids within this response has been the subject of considerable recent research. In this study we have examined the effects of the corticosteroid prednisolone on the production of IL-1α and IL-1β by lipopolysaccharide (LPS) stimulated monocytes, and the ability of the monocyte conditioned media (MOCM) obtained under these conditions to induce human hepatoma HepG2 cells to produce serum amyloid A (SAA) and C-reactive protein (CRP). We also examined the production of SAA and CRP by HepG2 cells exposed to different combinations and concentrations of recombinant human (rh) IL-1α, rhIL-1β, rhIL-6, rhTNF-α, and prednisolone. The findings indicate: (1) Prednisolone substantially inhibits the production of both IL-1α and IL-1β by LPS stimulated monocytes. The MOCM from prednisolone treated monocytes induced less SAA and CRP production by HepG2 cells. (2) IL-1α and IL-1β both induced CRP and SAA synthesis by HepG2 cells, but only in the presence of IL-6. Il-1β was the more potent inducer for SAA production, but for CRP production IL-1α and IL-1β were equivalent. (3) Prednisolone enhances the production of SAA by HepG2 cells, but does not enhance the production of CRP. (4) TNF-α in the presence or absence of IL-6 and/or prednisolone did not induce the production of SAA

or CRP by HepG2 cells. These findings offer a tenable solution to a disparate production of SAA compared to CRP in corticosteroid treated CF patients.

INTRODUCTION

Acute phase proteins are synthesized by the liver as part of a non-specific response to tissue damage or infection (Schultz and Arnold, 1990). Most investigators agree that the purpose of the acute phase response is to confine the source of inflammation, limit autolytic damage by phagocytic cells, and ultimately lead to the repair of the damaged tissue (Stadnyk et al., 1990). Regulation of the synthesis of acute phase proteins is modulated by a very complex network of cytokines which can act independently or in concert with each other and with endocrine hormones and glucocorticoids to inhibit or stimulate the production of acute phase proteins (Mackiewicz et al., 1991). The major cytokines involved in the regulation of the synthesis of acute phase proteins are interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), and leukemia inhibitory factor (LIF) (Raynes et al., 1991). Until recently, it was thought that IL-1α and IL-1β had identical activities in promoting the production of the acute phase reactants, binding to the same receptor and triggering the same responses. Recent observations, however, have raised questions as to the activities and binding characteristics of IL-1α and IL-1β. IL-1α and IL-1β have only a 26% homologous amino acid sequence (Endres et al., 1987), and it was recently demonstrated that the two IL-1 genes have very little in common (Mora et al., 1990). In addition, the IL-1β gene was shown to be clearly dominant in human monocytes, and to have a strong enhancer

allowing high levels of expression (Mora et al., 1990), whereas IL-1α was primarily produced by keratinocytes (Ansel et al., 1988) and T cells (Tartakovsky et al., 1986).

The receptor expression and cellular distribution for IL-1α and IL-1β also differ significantly. Scapigliati, et al., (1989) demonstrated that murine EL4-6.1 thymoma cells possessed more that 22,000 receptors per cell for IL-1α, (k_d 1.0 nM), but less than 3,000 receptors for IL-1β (k_d 0.36 nM). Human B lymphoma RAJI cells, however, have nearly 8 times as many receptors for IL-1β (2400 receptors per cell, k_d 2.2nM) as for IL-1α (316 receptors per cell, k_d 0.13nM). Scapigliati also demonstrated that unlabelled IL-1β was able to competitively displace both radiolabelled IL-1α and IL-1β from RAJI cells, while unlabelled IL-1α was unable to displace radiolabelled IL-1β. The receptor which predominates on T cells and preferentially binds IL-1α has a molecular weight of 80 kDa, and the B cell receptor, which binds only IL-1β, has a molecular weight of 68 kDa. Ghiara et al. (1990) recently demonstrated that HepG2 cells express both receptors, approximately 1300 of the IL-1α binding receptors (k_d 0.18 nM) and 417 of the IL-1β receptors (k_d 0.29 nM). HepG2 cells thus possess two structurally different IL-1 receptors with distinct binding properties for IL-1α and IL-1β (Ghiara et al., 1990).

The acute phase proteins exhibit different magnitudes of increase following stimulus (Ganapathi et al., 1991). In

humans, SAA and CRP are the major acute phase proteins measured as indicators of inflammation. Their plasma concentrations may increase as much as several thousand-fold over normal physiological concentrations (Ganapathi et al., 1991), with SAA reaching concentrations as high as 1 mg/ml during acute inflammation (Schultz and Arnold, 1990). Plasma CRP levels have been used as an aid in diagnosis of both primary conditions and superimposed infections, and as a measure of disease activity in patients suffering from rheumatoid arthritis and other inflammatory disorders, malignancies, etc. (Schultz and Arnold, 1990; Kushner and Mackiewicz, 1987). Serum amyloid A level has similar clinical value, and in some instances has been shown to be a more sensitive indicator of inflammation than CRP (Marhaug et al., 1983; Maury and Teppo, 1984; Chambers et al., 1991; Smith et al., 1992). Plasma levels of SAA and CRP correlate with pulmonary inflammation secondary to lung infections in CF patients (Marhaug et al., 1983; Smith et al., 1992). We recently reported an apparent disparate production of SAA compared to CRP in CF patients receiving systemic steroidal anti-inflammatory therapy when they developed P. aeruginosa lung infections, wherein the ratio of SAA to CRP production was significantly increased (3-fold) when compared to patients not receiving corticosteroid therapy (Smith et al., 1992).

These observations prompted us to examine the effects of prednisolone (the active metabolic product of the

corticosteroid prednisone) on the production of SAA and CRP in HepG2 cells stimulated with IL-1α, IL-1β, or TNF-α in combination with each other and/or IL-6. The effect of prednisolone on the production of IL-1α and IL-1β by peripheral blood monocytes when stimulated with lipopolysaccharide (LPS) from E.coli or with killed P. aeruginosa was examined. The ability of the monocyte conditioned media (MOCM) obtained under these conditions to induce HepG2 cells to produce SAA and CRP was also evaluated.

MATERIALS AND METHODS

Cytokines Recombinant human (rh) IL-1α and rhIL-1β were obtained from the Biological Response Modifier Program, National Cancer Institute, Frederick, MD. rhIL-6 was a very kind gift from Drs. May and Sehgal, Rockerfeller University, New York, NY. rhTNF-α was from Genentech (South San Francisco, CA).

IL-1 monoclonal antibodies All antibodies for IL-1α and IL-1β assays, as well as neutralizing antibodies for IL-1α and IL-1β, were very kind gifts from Dr. John Kenney, Syntex Laboratories, Palo Alto, CA.

Cell Culture All cells were maintained in prepared RPMI-1640 medium (Gibco, Grand Island, NY) (with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine,

penicillin/streptomycin, and fungizone (all from Gibco)) and were incubated at 37°C in an atmosphere of 5% CO2.

MOCM preparation Fresh heparinized whole blood was obtained by venipuncture from healthy volunteers. The mononuclear cell fraction was separated by gradient centrifugation on Histopaque-1077 (Sigma, St Louis, MO). Monocytes were separated by adherence to plastic tissue culture flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in prepared RPMI-1640 for two hours. Nonadherent cells were removed and discarded. Adherent cells were washed twice, then placed in fresh medium, and incubated overnight at 37°C. These quiescent adherent cells were washed once more, placed in fresh medium, then stimulated with either 10 μg/ml LPS from E. coli (Difco, Detroit, MI) or a killed suspension of P. aeruginosa. After 48 hours conditioned media was collected, centrifuged, filter sterilized, and stored at -20°C until used. Cells prepared in this manner appeared morphologically to be >95% monocytes. Viability of monocytes was routinely assessed by trypan blue exclusion after 48 hour incubation with or without LPS, in the presence or absence of prednisolone, and was always greater than 90%.

Neutralization studies Monoclonal antibodies which have been shown to specifically neutralize IL-1α (ILA8-H12) or IL-1β (ILB1-H34.3) without cross-reacting with IL-2, IL-4, IL-6, or

TNF (Kenney et al., 1987; Sandborg et al., 1989), or appropriate control material, were added to MOCM at 5 μg/ml, mixed thoroughly, and incubated at 4°C for 2 hours. This amount of antibody was sufficient to neutralize up to 5 ng/ml IL-1α or IL-1β (Sandborg et al., 1989). MOCM with both antibodies added at 5 μg/ml was also prepared in the same manner.

HepG2 Cells HepG2 human hepatoma cells were a gift from Dr. Dean Tuma, Veterans Administration Hospital, Omaha, NE. Cells were passaged every seven days, and were plated at approximately 5 x 10^4 cells per well in 24 well tissue culture plates (Falcon) or at 5 X 10^5 cells per 25 cm^2 tissue culture flask (Falcon). Hepatoma cells were stimulated with 20% MOCM or with various combinations of cytokines and/or corticosteroids on the fifth day after plating. After 48 hours medium was removed and assayed for SAA and CRP, or was frozen at -20°C till assayed.

IL-1 Assay Vinyl assay plates (Costar, 2596, Cambridge, MA) were coated with either purified ILA9-H18.2 or ILB1-H6.81 diluted in phosphate-buffered saline (PBS) at 1.5 μg/well overnight at 4°C. After washing in 0.1% BSA/0.05% Thimerosal/PBS, non-specific binding sites were blocked by incubating for one hour at room temp with 5% non-fat dry milk/0.05% Thimerosal/PBS (200 μl/well). After washing, 50 μl

MOCM or standards which had been appropriately diluted in 1% non-fat dry milk/0.05% Thimerosal/PBS were added with 50 μl of biotinylated ILA8-H12 (2μg/ml) or ILB1-H67 (1μg/ml), and incubated for 2 hr at room temp. After washing, 100μl of a 1:3000 dilution of peroxidase conjugated strepavidin (Zymed Labs, South San Francisco, CA) was added and incubated for 1 hr. After washing, 100 μl/well of peroxidase substrate solution was added and incubated in the dark for 30 minutes. Tetramethyl benzidine (TMB) substrate solution (Kirkegaard and Perry, Gaithersburg, MD) was used for the IL-1α assay, and orthophenylene diamine (OPD) substrate solution (1 mg/ml OPD/0.03% H_2O_2/0.1 M citrate buffer, pH 4.9) was used for the IL-1β assay. Absorbance was measured at 450 nm for the IL-1β assay, and at 630 nm for the IL-1α assay. Sensitivities were approximately 15 pg/ml (Kenney et al., 1987; Sandborg et al., 1989).

SAA assay The SAA assay was a modification of the recently published assay of McDonald et al., (1991). Immulon II ELISA plates (Dynatech, Chantilly, VA) were coated with 1 μg/well purified anti-SAA monoclonal antibody 6B10 in pH 9.6 carbonate buffer for one hour at 37°C. After washing with PBS/0.05% Tween-20, 90 μl (containing 0.4 μg) of an alkaline phosphatase conjugated anti-SAA monoclonal antibody, 5G6 (specific for a separate, non-overlapping epitope of SAA) was added to the 6B10 coated well, immediately followed by test samples (10 μl)

or appropriately diluted (in PBS/0.05% Tween-20) standards (Smith and McDonald, 1991). After a one hour incubation at 37°C, the plate was washed with PBS/0.05% Tween-20, and 100 µl of 1 mg/ml alkaline phosphatase substrate (Sigma 104, St Louis, MO) dissolved in 10% diethanolamine/0.01% $MgCl_2 \cdot 6H_2O$, pH 9.8, was added. After a 1 hr incubation at 37°C, absorbance was at 405 nm was measured. Sensitivity was approximately 2 ng/ml. The antibodies, purified SAA, standards, and the SAA assay are available from BioSource International (Camarillo, CA).

CRP assay CRP in tissue culture fluid was assayed using the procedure described by Janssen et al. (1986). Vinyl assay plates (Costar, 2596, Cambridge, MA) were coated with rabbit anti-human CRP (DAKO, Carpinteria, CA) diluted 1:500 in 0.1 M, pH 9.6 carbonate buffer (100 µl/well) for 45 min at 37°C. Plates were washed with 0.05 M Tris-HCl/0.3M NaCl/0.05% Tween-20, pH 8.0 (CRP wash buffer), tissue culture supernatant or standards appropriately diluted in CRP wash buffer was added, and the plates were incubated an additional 45 min at 37°C. Plates were washed and horseradish peroxidase conjugated rabbit anti-human CRP (Dako) diluted 1:500 in CRP wash buffer was added, and incubated for 30 min at 37°C. Plates were washed, then developed as described previously for the IL-1β assay. Sensitivity of the assay was approximately 0.5 ng/ml.

RESULTS

Effect of Prednisolone on production of IL-1α and IL-1β by monocytes.

The effect of prednisolone on the ability of monocytes to produce IL-1α and IL-1β in our culture system is shown in Table 1. IL-1α and IL-1β were undetectable in MOCM in the absence of LPS stimulation, whether or not prednisolone was added. Prednisolone added to monocytes at a final concentration of 1.0 μM six hours prior to stimulation with E. coli LPS reduced the production of IL-1α and IL-1β to 47% and 46% respectively when compared to monocytes stimulated with LPS in the absence of prednisolone. When prednisolone was added at the time of LPS stimulation, IL-1α and IL-1 production were 54% and 58% respectively, which indicated that the inhibition noted with prednisolone was not dependent on preincubation of the monocytes with prednisolone prior to LPS stimulation. Comparable results were obtained by stimulation of monocytes with killed P. aeruginosa (data not shown).

Effect of prednisolone on the ability of MOCM to induce HepG2 cells to produce SAA and CRP

Since there would be carry-over prednisolone in the conditioned media from monocytes treated with prednisolone, it was important to first determine the effect of prednisolone on the production of CRP and SAA by HepG2 cells stimulated with MOCM. In four experiments prednisolone (1.0 μM) was added

along with MOCM obtained from LPS stimulated monocytes which were not treated with prednisolone. The added prednisolone had no effect on CRP production, but induced HepG2 cells to produce a 6-fold greater concentration of SAA. CRP concentrations were unaffected by prednisolone addition (11.3 ± 2.8 ng/ml in the absence of prednisolone, 11.8± 1.2 ng/ml in the presence of 1.0 μM prednisolone) while SAA production by HepG2 cells increased from 52 ng/ml (SD 16 ng/ml) to 312 ng/ml (SD 100 ng/ml) when prednisolone was added to 1.0 μM. In order to assure that prednisolone had no independent effect on the production of CRP and SAA by HepG2 cells, prednisolone was added without MOCM or with MOCM from monocytes which received no LPS stimulation; neither of these induced measurable production of either CRP or SAA.

These data showed that in order to make direct comparisons of the biological activities of the three monocyte conditioned media (1. prednisolone added concurrently with LPS, 2. prednisolone added 6 hours prior to LPS, or 3. no prednisolone added), it was important to equalize the enhancing effect of prednisolone on SAA production by HepG2 cells. Therefore, each of the filter sterilized MOCM was diluted to 20% in prepared RPMI 1640 media and brought to 1.0 μM prednisolone prior to testing for their ability to induce CRP and SAA production by HepG2 cells. Consistent with measured levels of IL-1α and IL-1β, MOCM from monocytes treated with prednisolone induced significantly less production of CRP and SAA than MOCM

obtained from monocytes which were not treated with prednisolone (36% and 28% respectively for SAA and CRP) (Table 2). MOCM obtained from monocytes pretreated with prednisolone six hours prior to LPS stimulation induced even less CRP and SAA production (23% for each) by HepG2 cells.

Effect of addition of IL-1α and IL-1β neutralizing antibodies to MOCM on induction of HepG2 cells to produce SAA and CRP.

Treatment of MOCM with neutralizing monoclonal antibodies to IL-1α and IL-1β reduced its ability to induce the production of both CRP and SAA by HepG2 cells. Individually, each antibody neutralized a portion of the CRP and SAA production activity of MOCM, and when added together, essentially all CRP and SAA production activity was neutralized. This demonstrated that either IL-1α or IL-1β must be present in MOCM in order for HepG2 cells to produce CRP or SAA, and that each was biologically active in our system (figure 1).

Effect of recombinant cytokines and prednisolone on the production of CRP and SAA by HepG2 cells.

rhIL-1α, rhIL-6, and prednisolone.

Nearly confluent HepG2 cells were stimulated with different concentrations of rhIL-1α and rhIL-6 either in the absence of prednisolone or in the presence of 0.1 μM prednisolone or 1.0 μM prednisolone. CRP production in this system was maximum at 5.0 ng/ml rhIL-6, and 25 ng/ml rhIL-1α (figure 2a). Addition

of rhIL-6 at concentrations above 5.0 ng/ml caused no further increase in CRP production. Presence of prednisolone had no effect on production of CRP (data not shown).

Maximum SAA production was achieved with rhIL-6 at 5.0 ng/ml and rhIL-1α at 25 ng/ml (figure 2b). In contrast to CRP production, SAA production by HepG2 cells was increased by 3-fold with 0.1μM prednisolone, (data not shown) and up to 5-fold with 1.0 μM prednisolone (figure 2c). Addition of rhIL-6 at concentrations above 5.0 μM or prednisolone at concentrations above 1.0 μM caused no further increase in SAA production.

rhIL-1β, rhIL-6, and prednisolone

When rhIL-1β was titrated in conjunction with different levels of rhIL-6 to stimulate CRP production by HepG2 cells, maximal activity was found at 5.0 ng/ml of rhIL-6, and 25 ng/ml of rhIL-1β (figure 3a). As with rhIL-1α, addition of prednisolone caused no further increase in production of CRP (data not shown).

rhIL-1β and rhIL-6 combined at concentrations of 25 ng/ml of rhIL-1β and 2.5 ng/ml of rhIL-6 caused maximal production of SAA (figure 3b), at levels five times higher than the maximum reached with rhIL-1α and rhIL-6 (160 ng/ml as compared to 33 ng/ml). This activity was further enhanced with prednisolone, and reached 3-fold higher concentrations with 0.1 μM

prednisolone (data not shown), and 5-fold higher concentrations with 1.0 μM prednisolone (figure 3c).

Effect of rhTNF-α

rhTNF-α was added to HepG2 Cells at 750, 200, 50, and 20 units per ml in the presence or absence of rhIL-6 (2.5 ng/ml). rhTNF-α did not induce any measurable production of CRP or SAA in our system. The experiments were repeated with rhTNF-α and rhIL-6 in the presence of 1.0 μM prednisolone with the same results, i.e., no measurable production of CRP or SAA.

Competitive activities of rhIL-1α and rhIL-1β.

In the presence of 2.5 ng/ml rhIL-6 and 1.0 μM prednisolone, rhIL-1α and rhIL-1β showed nearly equivalent activity in their ability to induce HepG2 cells to produce CRP (figure 4a). Maximum CRP production in this system was achieved with 30 ng/ml each of rhIL-1α and rhIL-1β. rhIL-1α caused CRP to be produced in a dose dependent fashion such that approximately 70% of maximum production occurred with 30 ng/ml rhIL-1α (figure 4a). rhIL-1β under the same conditions induced approximately 80% of maximum production of CRP, also in a dose dependent manner (figure 4a).

In contrast, in the same system rhIL-1α induced only approximately 25% of maximum SAA production in the absence of rhIL-1β, while rhIL-1β at 20 ng/ml and 30 ng/ml induced near maximum (>90%) SAA production in the absence of rhIL-1α. Thus

it appeared that for SAA production rhIL-1β was the more potent stimulant, and that high levels of rhIL-1β ($\geq$ 20 ng/ml) abrogated further SAA production induced by rhIL-1α (figure 4b).

DISCUSSION:

The findings of this paper offer a tenable solution to the disparate production of SAA vs CRP observed in cystic fibrosis patients receiving corticosteroid anti-inflammatory therapy (Smith et al., 1992). Corticosteroids decreased the production of IL-1α and IL-1β by cultured monocytes, thus decreasing the signal which "drives" the production of the acute phase proteins by HepG2 cells. However, it appears that corticosteroids can amplify this decreased IL-1 signal to increase the production of SAA several-fold, while production of CRP does not appear to have the same amplification mechanism . Castell et al., (1988) demonstrated comparable results when examining the effect of dexamethasone on IL-6 stimulated primary cultures of human hepatocytes. In their experiments, the production of SAA was increased 6-fold by the presence of 0.1μM dexamethasone, while the production of CRP was unaffected. They found that other acute phase proteins were variously affected by dexamethasone; haptoglobin production was doubled, while the production of fibrinogen was unaffected. These reports lend credence to the concept that

corticosteroids may be intimately involved in the differential regulation of production of acute phase proteins.

Ghezzi and Sipe (1988) showed that the influence of corticosteroids on the production of acute phase proteins was not isolated to in-vitro experiments. When mice were injected with 1mg dexamethasone 30 minutes prior to stimulation with LPS, the response as measured by production of SAA was decreased by approximately 70%. This indicated that corticosteroids dampened at least that portion of the acute phase response which resulted in SAA synthesis. However, when IL-1 or TNF was injected into animals that were pretreated with 1.0 mg dexamethasone SAA production was increased by 2.5-fold and 1.5-fold respectively compared to animals that received IL-1 or TNF alone.

The studies conducted with MOCM showed: (1) Monocytes stimulated with LPS or killed P. aeruginosa produced a MOCM that contained both IL-1α and IL-1β, and the concentrations of IL-1α and IL-1β in the MOCM from monocytes treated with prednisolone was significantly decreased. (2) MOCM from this system induced the production of both SAA and CRP by HepG2 cells, and part of that induction capability was removed with neutralizing antibody to either IL-1α or IL-1β. (3) Essentially a complete loss of production of both SAA and CRP was observed when HepG2 cells were stimulated with MOCM in which both IL-1α and IL-1β had been neutralized. This latter finding ruled out any major involvement of TNF or other

cytokines for LPS stimulated MOCM induction of SAA and CRP synthesis by HepG2 cells. In fact, when rhTNF-α was added to HepG2 cells with or without rhIL-6 present, no SAA or CRP production was detected. This is in contrast to the findings of Ganapathi (1991) wherein TNF-α in the presence of IL-6 induced Hep3B cells to produce SAA, but not CRP, although he noted that such production seemed to vary dependent on the batch of TNF-α employed. It is conceivable that a different lot of rhTNF-α might have had activity in our system.

Checkerboard titrations with rhIL-1α and rhIL-6 or rhIL-1β and rhIL-6 at various concentrations of prednisolone revealed, in agreement with recent observations (Ganapathi et al., 1991; Ganapathi et al., 1988), that increased concentrations of either rhIL-1 or rhIL-6 led to an increase in the synthesis of both SAA and CRP by HepG2 cells, as long as a minimal amount of the other cytokine was available. Although rhIL-1β was more active than rhIL-1α in stimulating SAA production they were equally active for inducing the production of CRP.

Checkerboard titrations of rhIL-1α vs rhIL-1β at constant levels of rhIL-6 and prednisolone confirmed these observations. Maximum SAA production achieved with rhIL-1α was increased by addition of rhIL-1β, however the opposite was not true. This observation is consistent with the experimental data recently reported by Ghiara et al. (1990), that HepG2 cells possess two IL-1 receptors, one for IL-1α and one for IL-1β, and Scapigliati's findings (Scapigliati et al.,

1989) that IL-1β is able to displace radiolabelled IL-1α from its receptor, but that IL-1α is unable to displace radiolabelled IL-1β.

The finding that the activities of rhIL-1α and rhIL-1β in stimulating HepG2 cells to produce SAA and CRP were not identical was not unexpected. Raynes et al., (1991) had previously shown that HUH-7 hepatoma cells, in the presence of 0.1 μM dexamethasone, produced nearly twice as much SAA in response to maximal doses of IL-1α compared to maximal doses of IL-1β. Our findings indicate the opposite effect in HepG2 cells, i.e., that rhIL-1β is the more potent inducer of SAA synthesis. Given the low degree of homology between IL-1α and IL-1β (Endres et al., 1987), the differences in their synthesis (Ansel et al., 1988; Tartakovsky et al., 1986; Hogquist et al., 1991), and genetic structure and regulation (Mora et al., 1990), as well as the existence of two receptors with different binding affinities and molecular weights (Scapigliati et al., 1989; Ghiara et al., 1990), it is probable that different activities will be delineated for IL-1α and IL-1β, even though obviously many overlapping activities exist.

The findings of this study suggest that there may be significant differences in the modulation of different acute phase proteins by different combinations of cytokines and corticosteroids. SAA and CRP levels normally increase concurrently in disease, although Maury and Teppo (1984)

showed that is not always the pattern followed. The acute phase response is an integrated response involving a number of cytokines (IL-1α, IL-1β, IL-6, TNF, LIF, etc), hormones such as insulin, and corticosteroids, prostaglandins, etc. These many factors may be produced in different combinations dependent upon the source of initiation, the pathophysiological conditions and modalities in patient therapy. These combinations of factors then set in motion a series of events among which are the differential expression of SAA and CRP.

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Table 1.

Effect of prednisolone on the production of IL-1α and IL-1β by LPS-stimulated monocytes.[a]

MOCM Preparation

	No LPS		10 μg/ml LPS		
	No pred	1 μM pred	No pred	1 μM pred $_{-6}$hrs[b]	1 μM pred t_o[c]
IL-1α (pg/ml)	<15	<15	280 ±68 (100%)	130 ±49 (47%)	150 ±54 (54%)
IL-1β (pg/ml)	<15	<15	840 ±100 (100%)	385 ±50 (46%)	490 ±56 (58%)

[a]MOCM from control and treated monocytes was assayed for IL-1α and IL-1β 48 hrs after LPS stimulation

[b]t_{-6}: Prednisolone was added 6 hrs prior to stimulation with LPS.

[c]t_o: Prednisolone was added at the same time as LPS stimulation.

Values are expressed as mean±SD for 3 experiments. Values in parentheses are % maximum production.

Table 2.

Production of SAA and CRP by HepG2 cells stimulated with MOCM produced in the presence or absence of prednisolone.[a]

	MOCM Preparation				
	No LPS		10 μg/ml LPS		
	No pred	1 μM pred	No pred	1 μM pred t_{-6}hrs[b]	1 μM pred t_o[c]
CRP (ng/ml)	< 0.5	< 0.5	12 ±3.2 (100%)	2.7 ±1.2 (23%)	3.4 ±1.5 (28%)
SAA (ng/ml)	< 2.0	< 2.0	318 ±53 (100%)	73 ±45 (23%)	112 ±35 (35%)

[a]Tissue culture fluid was assayed for SAA and CRP 48 hrs after addition of MOCM.

[b]t_{-6}: Prednisolone was added 6 hrs prior to stimulation with LPS.

[c]t_o: Prednisolone was added at the same time as LPS stimulation.

Values are expressed as mean±SD for 3 experiments. Values in parentheses are % maximum production.

Figure 1

% Maximum
0
20
40
60
80
100
CRP
VAS
Both antibodies
Anti IL1-B
Anti IL1-α
No antibody
Both antibodies
Anti IL1-B
Anti IL1-α
No antibody

Figure 2

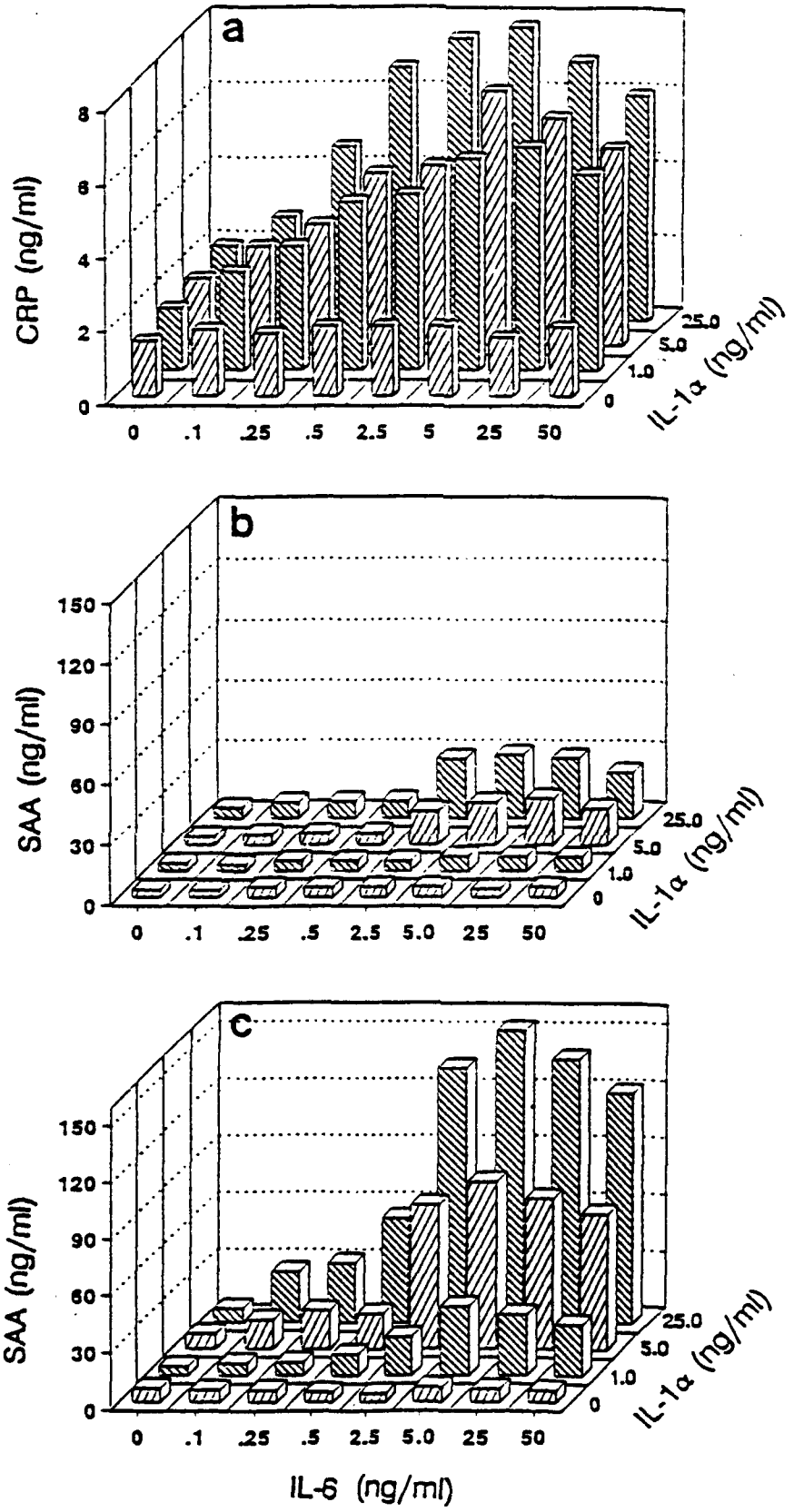

a
CRP (ng/ml)
0
2
4
6
8
0 .1 .25 .5 2.5 5 25 50
IL-1α (ng/ml)
0
1.0
5.0
25.0
b
SAA (ng/ml)
0
30
60
90
120
150
0 .1 .25 .5 2.5 5.0 25 50
IL-1α (ng/ml)
0
1.0
5.0
25.0
c
SAA (ng/ml)
0
30
60
90
120
150
0 .1 .25 .5 2.5 5.0 25 50
IL-6 (ng/ml)
IL-1α (ng/ml)
0
1.0
5.0
25.0

Figure 3

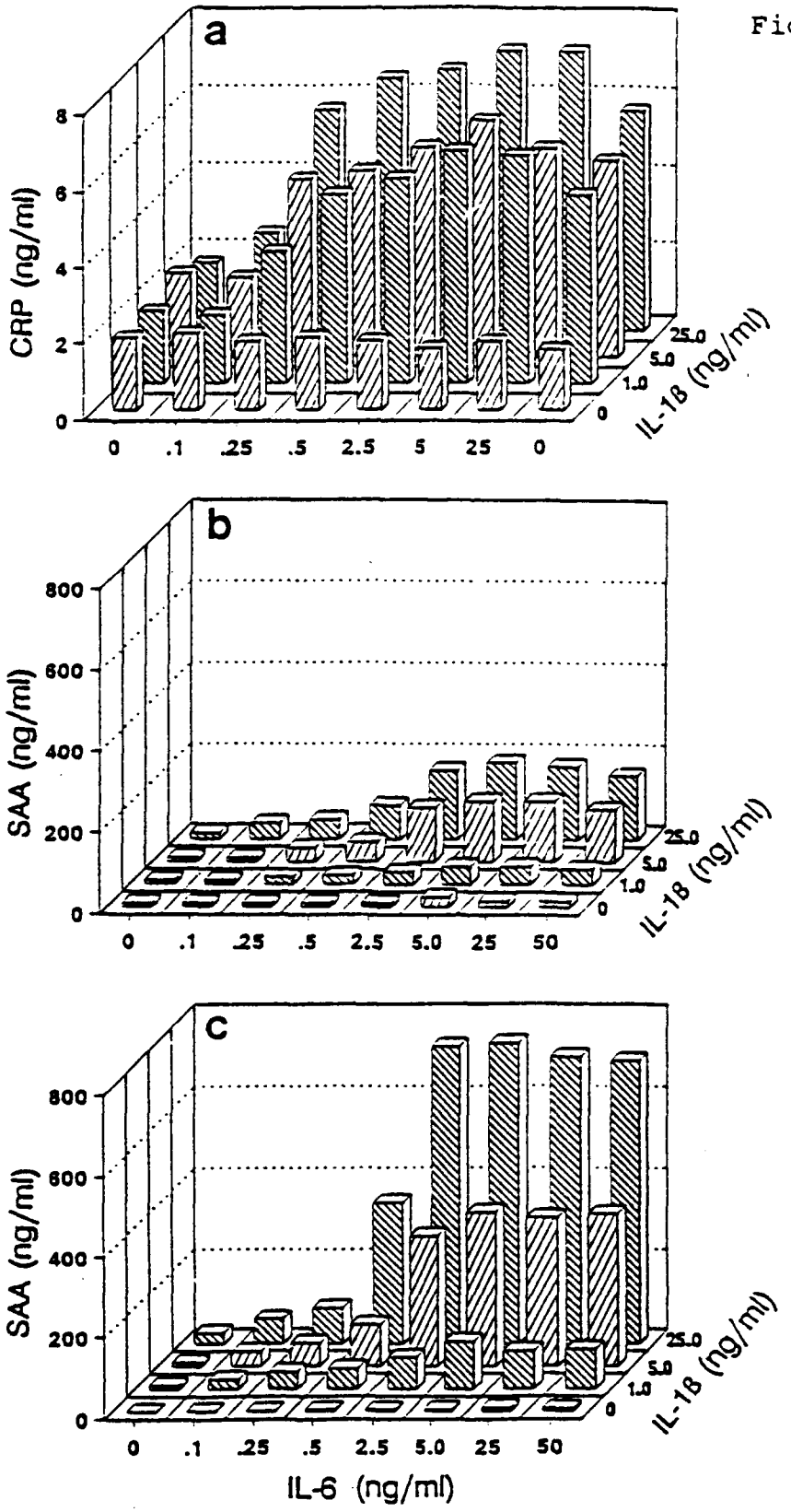
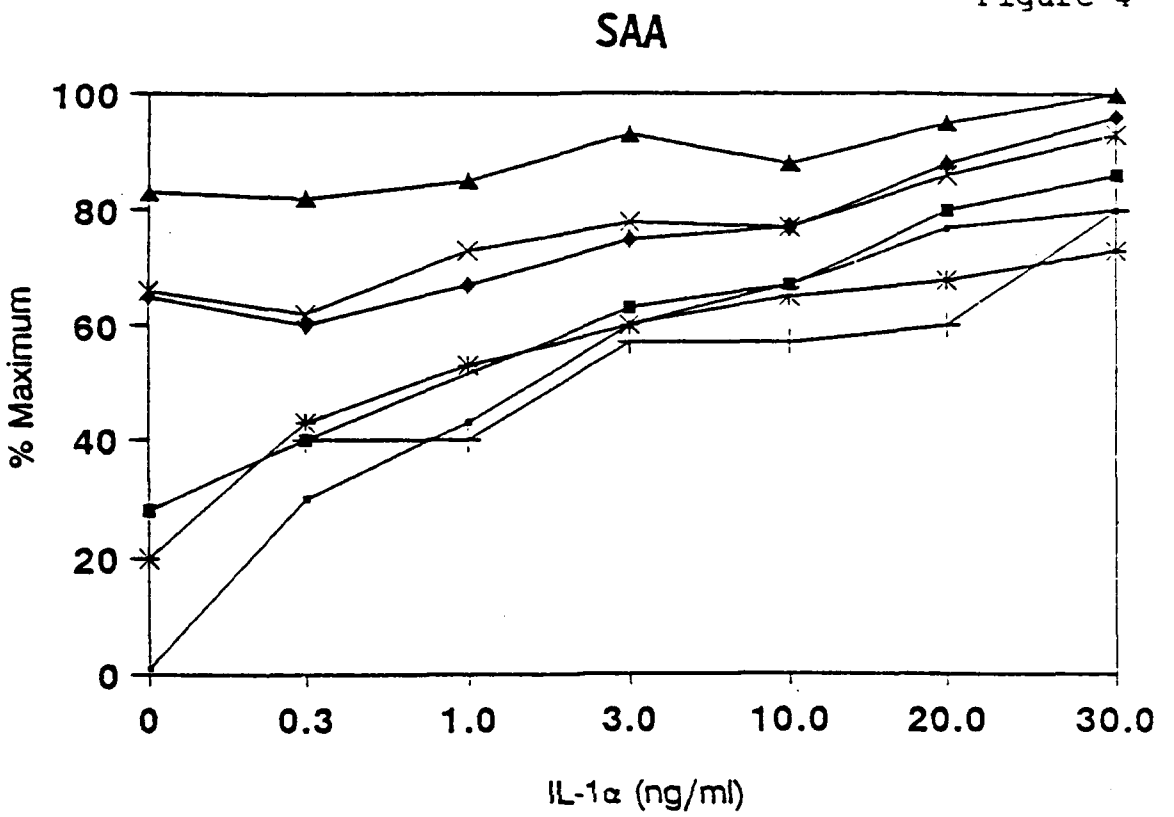
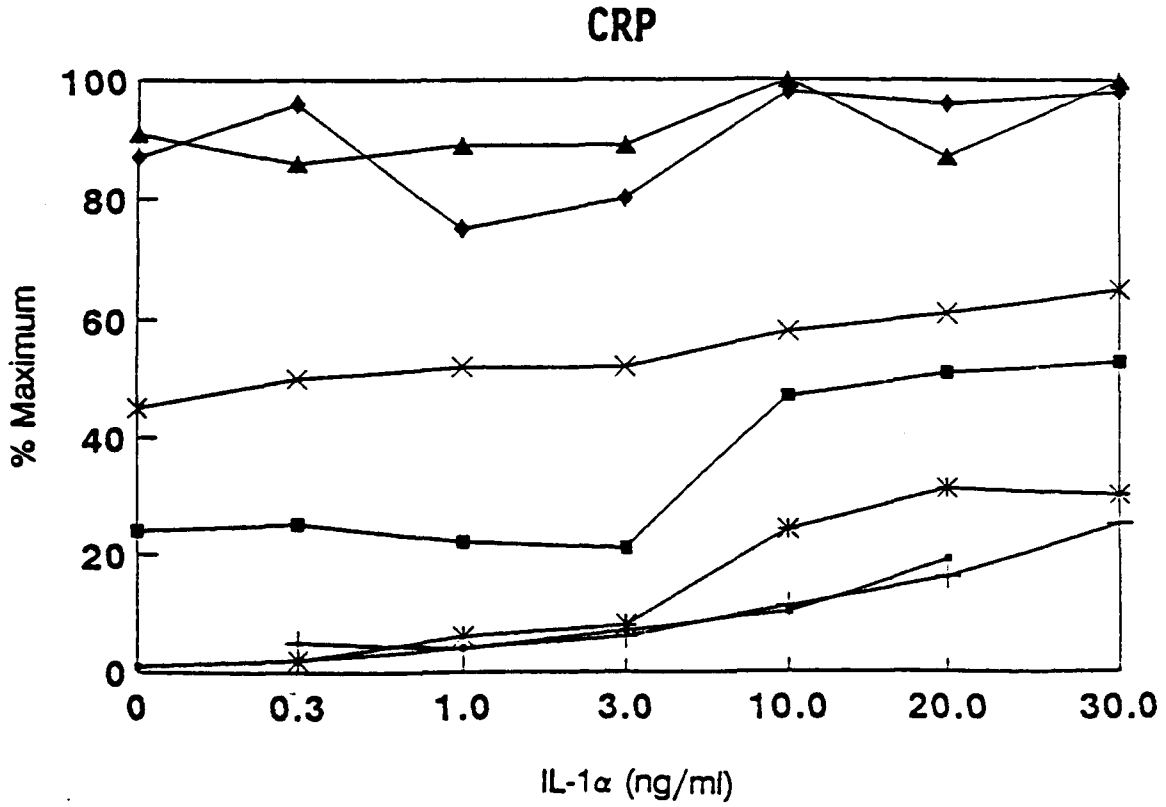

a
CRP (ng/ml)
0
2
4
6
8
0 .1 .25 .5 2.5 5 25 0
0
1.0
5.0
25.0
IL-18 (ng/ml)
b
SAA (ng/ml)
0
200
400
600
800
0 .1 .25 .5 2.5 5.0 25 50
0
1.0
5.0
25.0
IL-18 (ng/ml)
c
SAA (ng/ml)
0
200
400
600
800
0 .1 .25 .5 2.5 5.0 25 50
IL-6 (ng/ml)
0
1.0
5.0
25.0
IL-18 (ng/ml)

Figure 4


SAA
100
80
60
40
20
0
% Maximum
0
0.3
1.0
3.0
10.0
20.0
30.0
IL-1α (ng/ml)


CRP
100
80
60
40
20
0
% Maximum
0
0.3
1.0
3.0
10.0
20.0
30.0
IL-1α (ng/ml)

Legends

Figure 1. Effect of treatment of MOCM with IL-1α and IL-1β neutralizing antibodies on the induction of SAA and CRP production by HepG2 cells. Monoclonal anti-IL-1α and anti-IL-1β were added to MOCM at 5 μg/ml final concentration. After incubation at 4°C the treated MOCM was used to induce HepG2 cells to produce SAA and CRP. The horizontal lines are the mean values obtained from two experiments, are the actual values.

Figure 2. Production of CRP and SAA by HepG2 cells induced with different concentrations of rhIL-1α, rhIL-6, and prednisolone. Concentrations of rhIL-6 were varied from 0 to 50 ng/ml and concentrations of rhIL-1α were varied from 0 to 25 ng/ml. Values expressed are the means of 3 experiments. Interexperimental variation was less than 25%. a. CRP produced in the absence of prednisolone (prednisolone had no effect on CRP production, see text). b. SAA produced in the absence of prednisolone. c. SAA produced in the presence of 1.0 μM prednisolone.

Figure 3. Production of CRP and SAA by HepG2 cells induced with different concentrations of rhIL-1β, rhIL-6, and prednisolone. Concentrations of rhIL-6 were varied from 0 to 50 ng/ml and concentrations of rhIL-1α were varied from 0 to 25 ng/ml. Values expressed are the means of 3 experiments. Inter-experimental variation was less than 25%. a. CRP produced in the absence of prednisolone (prednisolone had no effect on CRP production, see text). b. SAA produced in the absence of prednisolone. c. SAA produced in the presence of 1.0 μM prednisolone.

Figure 4. Production of CRP and SAA by HepG2 cells induced with different concentrations of rhIL-1α and rhIL-1β, and optimal rhIL-6 and prednisolone concentrations. rhIL-1α and rhIL-1β concentrations varied from 0 to 30 ng/ml while rhIL-6 concentration was held constant at 2.5 ng/ml, prednisolone at 1.0 μM. Values expressed are means of % maximum production measured in three experiments. Interexperimental variation was less than 25%. a. CRP produced. b. SAA produced.

- IL-1β = 0
- IL-1β = 0.3 ng/ml
- IL-1β = 1.0 ng/ml
- IL-1β = 3.0 ng/ml
- IL-1β = 10.0 ng/ml
- IL-1β = 20.0 ng/ml
- IL-1β = 30.0 ng/ml

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